EFFECT OF BIOMASS GENERATED PRODUCER GAS, METHANE AND PHYSICAL PARAMETERS ON PRODUCER GAS FERMENTATIONS BY *CLOSTRIDIUM* STRAIN P11

By

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CHAPTER 1

INTRODUCTION

Fossil derived chemicals were the main source of feedstock for the manufacture of numerous products during the industrial era (Rogers et al. 2006), but the amounts of petroleum, natural gas and coal are decreasing at an alarming rate (Huber et al. 2006). Coupled with this are many factors like global warming, political instability of oil producing countries, and concerns over national security, which has resulted in the increased effort to look for renewable and biological methods of production of chemicals and fuels (Lashof and Ahuja 1990; Rogers et al. 2006). The Energy Information Administration (EIA) has indicated that in the United States about two-thirds of the oil imported is consumed by the transportation sector (Anonymous 2009a). Putsche and Sandor (1996) indicated that in 1990, 97% of transportation fuel was petroleum based, showing the strong dependence of the US economy on oil. Thus, there is a necessity to increase the energy options for transportation sector (Wyman 1996). This is the reason for the search of alternative fuels such as ethanol, biodiesel and butanol from renewable sources. Among the many options of alternative fuel, ethanol has numerous desirable properties of a good fuel such as high heat of vaporization, low flame temperature, greater gas volume change, high specific energy and high octane content, and in optimized spark ignition engines, ethanol can achieve 15% higher efficiency compared to

gasoline (Wyman 1996).

Biomass derived feedstocks are not only renewable but also carbon neutral and have the potential to replace significant amounts of fossil fuel consumption (Khanal 2008). Primary agricultural crops such as sugarcane and corn are the most important feedstocks for bioethanol production (Tsai et al. 2009b). Industrial production of bioethanol has been successfully demonstrated in the past using these feedstock's in Brazil (sugarcane) and the United States (Reddy et al.). As shown in Fig. 1.1, ethanol production increased drastically after 2005 and reached 9 billion gallons in 2008 (Anonymous 2009b) during which most of the ethanol production was from corn (Urbanchuk 2007). This tremendous growth in the use of corn is underscored by the aid of tax credits for the biofuel producers (Anonymous 2009c). Consequently, corn based ethanol production is projected to saturate over the next decade (Anonymous 2009c; Urbanchuk 2007). Besides all this, the use of corn for bio-ethanol production has raised numerous problems like the food versus fuel debate, availability of land to grow corn dedicated to biofuel production and the amounts of water needed for growing corn (Anderson et al. 2008). Thus, the new renewable fuel standard requires production of 0.1 billion gallons of cellulosic ethanol by 2010 and 16 billion gallons of cellulosic ethanol by 2022. Towards this effort companies such as Abengoa, Mascoma, Bluefire, and ICM Inc. have facilities under construction and have proposed to use feedstocks such as corn stover, wheat straw, barley straw, rice straw, switchgrass, wood waste, and urban waste (Anonymous 2009b).

Fossil Energy Ratio (FER) is one of the important metrics to compare the efficiency of different energy systems. It relates the energy in the fuel to the fossil

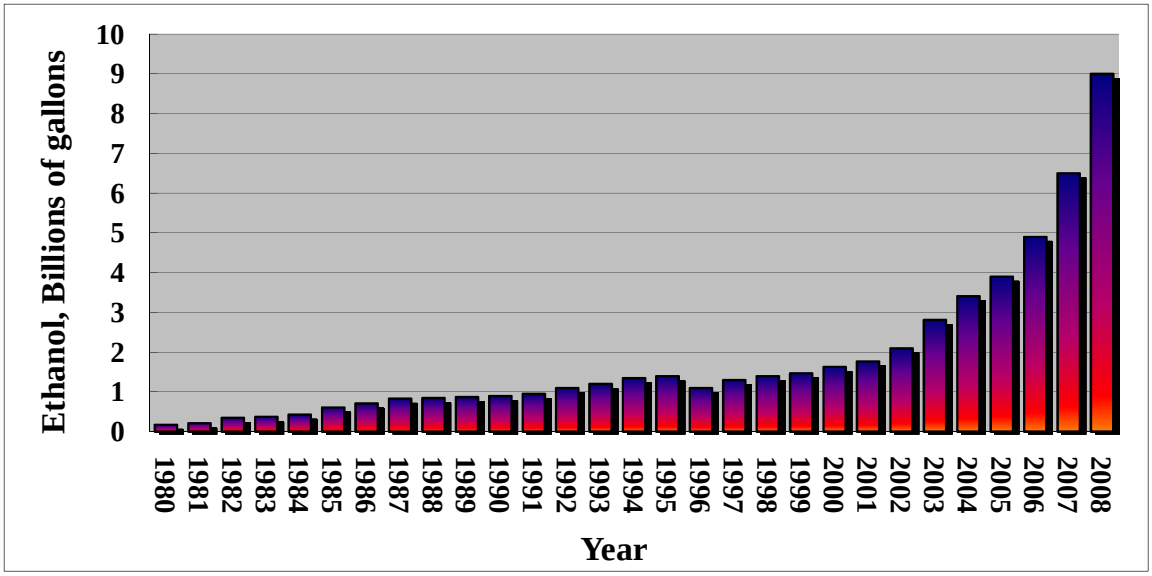

Figure 1.1 Historic US fuel ethanol production (Anonymous, 2009b).

energy utilized for its production (Wang 2005). FER of various energy systems are shown in Fig. 1.2. It is lucid from the graph that cellulosic ethanol can produce almost 10 times more energy compared to corn ethanol. The reason is that the energy inputs for irrigation, machinery and pesticide application are high for corn ethanol production. Besides this, a study at Argonne National Laboratory shows that the use of an 85% blend of cellulosic ethanol with gasoline (E85) will reduce the petroleum consumption by 70-71% and reduce the emissions of green house gases (GHG) by 68-102% (Wang et al. 1999). The automobile industry has released 7 million flex fuel vehicles that can use E85, but this is only 3% of the total number of vehicles on the road (Anonymous 2009b).

The conversion of lignocellulosic feedstocks into ethanol can be biologically achieved by two methods: hydrolysis-fermentation and syngas fermentation (Huber et al. 2006). In hydrolysis-fermentation, the complex structure of the plant is broken down by a pretreatment followed by an acid or enzymatic hydrolysis to release sugars which are then converted into ethanol by yeast or bacteria (Olofsson et al. 2008). Besides being an uneconomical, multistep-multiconversion process, it also suffers from the major drawback of not utilizing 25-30 % of the plant material, i.e. lignin (Tsai et al. 2009b). Syngas fermentation is a two step process which combines gasification and fermentation. In the first step, lignocellulosic feedstocks (switchgrass, miscanthus, corn stover, wheat straw, wood waste and urban waste) can be gasified to produce a combination of carbon monoxide, carbon dioxide and hydrogen gas (with other gases such as nitrogen, methane, ammonia, hydrogen sulfide) that is called synthesis gas, producer gas or syngas. This gas can then be fermented by anaerobic microbes such as *Clostridium ljundahlii, Clostridium autoethanogenum, Butyribacterium methylotrophicum, Clostridium carboxidivorans* and

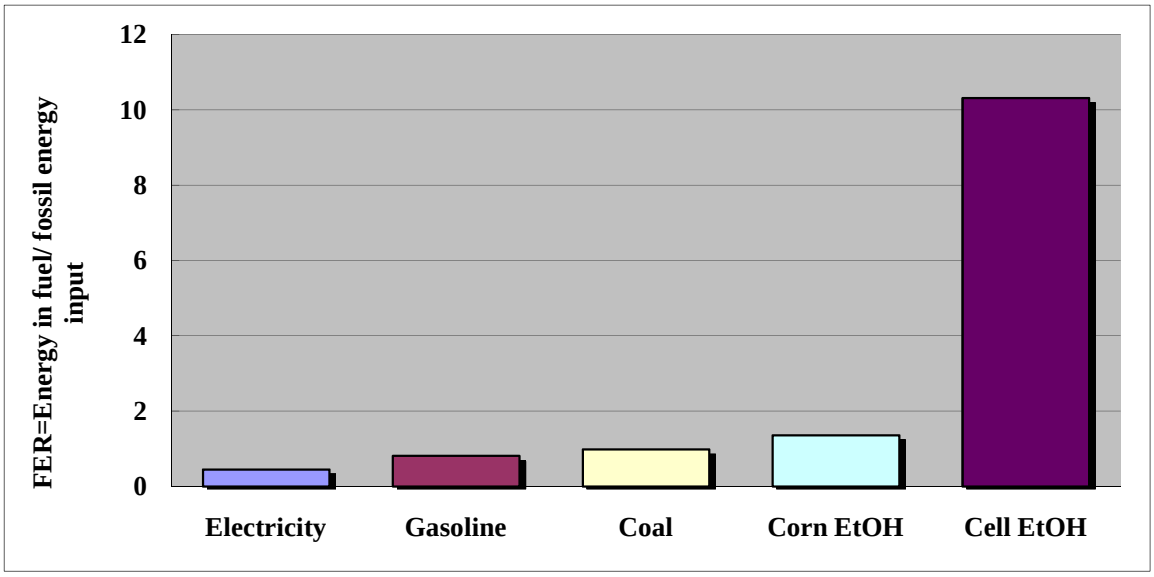

Figure 1.2 Fossil energy ratio (FER) of different processes (Wang 2005).

Clostridium strain P11 to form biofuels such as ethanol, isopropanol, butanol, hexanol and specialty chemicals such as acetic acid, butyric acid and hexanoic acid (Abrini et al. 1994; Grethlein et al. 1990). The gasification-fermentation process can utilize all the components of the biomass, which results in better conversion efficiency (McKendry 2002a). Furthermore, this process has the advantage of using different feedstocks (energy crops, agricultural wastes, industrial wastes and forest waste) depending on their availability and also using municipal waste, coal, natural gas, reformed gas, thus, making gasification-fermentation a flexible technology (Tsai et al. 2009a).

Almost all the studies on syngas fermentation that have been performed on topics such as design of novel reactors, optimization of media components and improving yields of ethanol employ synthetic gas (gases are mixed from commercially available gases) (Girbal et al. 1995a; Tsai et al. 2009b; Ungerman and Heindel 2008). However, the biomass generated producer gas contains many other components such as methane, acetylene, ethylene, ethane, nitric oxide, ammonia, hydrogen sulfide, benzene and toluene that could affect the overall fermentation process (Ahmed 2006). A study by Ahmed et al. (2006) on *Clostridium carboxidivorans* observed that nitric oxide in syngas can enhance ethanol formation, but tars present in syngas inhibit cell growth. However, there were no studies performed on biomass generated producer gas with P11. Thus, one of the objectives of this study was to observe the effect of producer gas made from switchgrass on P11 fermentations. It was hypothesized that the producer gas will affect P11 in a similar way as it did for *C. carboxidivorans*. This study will help understand the nuances of the fermentation problems that could be expected while using a biomass generated producer gas.

Producer gas fermentation is a relatively new technology and most of the transfer limited and the product (ethanol) is non-growth associated, it takes 15 to 45 days to complete a batch study in bottle reactors. Thus, an attempt to improve the efficiency of the process was performed by studying physical parameters such as agitation, temperature and amount of headspace gas. The amount of headspace (or available gas) was found as an important parameter by Frankman (2009). An experiment was conducted to further increase the headspace to improve the productivity of the process. In addition to headspace, agitation is an important parameter that affects mass transfer of gases. It was hypothesized that increasing agitation of bottles would increase ethanol yields. Finally, *Clostridium* bacteria are usually known to form spores by heat shock (Gapes et al. 2000). Jones et al. (1982) observed a positive correlation between sporulation and solventogenesis. This strategy of improving ethanol yields has not been conducted on P11. Thus, the hypothesis was that inducing heat shock would induce solventogenesis, which would eventually improve ethanol production. Hence, the study of the effect of physical parameters would help to improve solvent yields and productivities of bottle studies.

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CHAPTER 2

OBJECTIVES

The objectives of this research were as follows:

1. To study the effect of producer gas made from feedstocks such as switchgrass and corn gluten feed in P11 fermentations. To observe the effect of 5% methane in P11 fermentations.
2. To enhance solvent yields and productivities of producer gas fermentation bottle studies by evaluating the physical parameters such as headspace gas, agitation and temperature.

CHAPTER 3

REVIEW OF LITERATURE

3.1 Gasification process

Gasification is a very old technology. Producer gas was used to drive internal combustion engines as early as 1791 (Foley et al. 1983). Back in the 1940's, Sweden had over 70,000 "GENGAS" trucks (Klass 1998). The first commercial gasification plant (in England) used coal to produce a gas that was named "town gas" for street lighting purposes (Klass 1998). The first gasification plant in North America was situated at Baltimore (in 1816). By the end of the 19^{th} century and first half of the 20^{th} century, there were more than 1500 operational gas plants, but after the discovery of natural gas, the use of producer gas declined (Klass 1998). Interest in this technology grew again after the oil crisis in the 1970's and the increasing awareness on climate change and pollution caused by the use of fossil fuels (Klass 1998; Milne et al. 1998).

Gasification is a thermo-chemical conversion process in which carbonaceous materials (such as natural gas, naphtha, residual oil petroleum coke, coal or biomass) reacts with a gasification medium (such as air, oxygen and/or steam) at high temperatures (~600-1000 °C) to produce a mixture of gases called synthesis gas (syngas) or producer gas (El-Rub et al. 2004; Huber et al. 2006; Kumar et al. 2009; McKendry 2002b; Spath and Dayton 2003). The terms "syngas" and "producer gas" are used interchangeably, but

there is a difference between them based on the nitrogen content of the gas. Producer gas is generally obtained when air is used as a gasification medium and hence it has predominant levels of nitrogen and relatively smaller amounts of carbon monoxide, carbon dioxide, hydrogen and methane unlike syngas that is predominantly made up of carbon monoxide, hydrogen and carbon dioxide (Huber et al. 2006). The presence of nitrogen significantly decreases the heating value of the producer gas (Belgiorno et al. 2003). On the other hand, syngas is made predominantly of carbon monoxide (CO), carbon dioxide (CO_2) and hydrogen (H_2). The major components of producer gas are carbon monoxide, carbon dioxide, nitrogen and hydrogen, but it also contains methane, water, some higher hydrocarbons (acetylene, ethylene, ethane) and various contaminants like inorganic impurities (ammonia, HCN, hydrogen sulfide, ash), char particles (that is pure carbon and inert materials present in the feedstock) and organic impurities like tars (Belgiorno et al. 2003; Bridgewater 1994; El-Rub et al. 2004). Gasification can be used to convert low value feedstock's into heat, electricity, transportation fuels (hydrogen, Fisher-Tropsch diesel, synthetic gasoline) and chemicals like methanol and urea (El-Rub et al. 2004; Huber et al. 2006). An outline of the syngas conversion process (after gasification) with their end products is shown in Fig. 3.1.

Biomass such as energy crops, agricultural residues, food waste and forestry residues can be used to make producer gas. The advantage of biomass compared to coal is that biomass has highly oxygenated cellulose and hemicelluloses which makes it more reactive (Huber et al. 2006; Klass 1998; Kumar et al. 2009). Furthermore, there is more volatile content (2 to 2.3 times greater than coal) which in turn decreases the gasification temperature (Huber et al. 2006; Klass 1998; Kumar et al. 2009). The major disadvantage

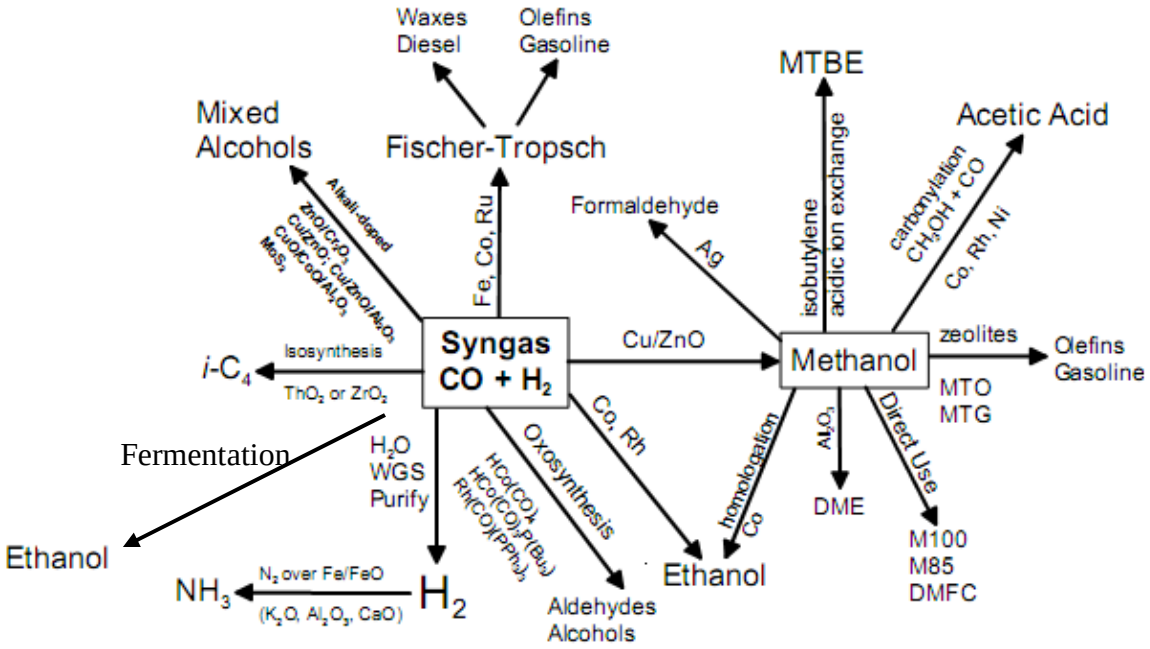

Figure 3.1 Syngas conversion process - Adapted from Spath and Dayton (2003).

of using biomass for gasification is the presence of alkali metals such as sodium, potassium, calcium, magnesium and others which cause problems such as slagging and fouling in gasification equipment (Huber et al. 2006).

The overall process of biomass gasification shown in Fig. 3.2, involves preprocessing of biomass, gasification and gas clean up. Preprocessing of the biomass is an upstream process that involves size reduction and drying. Size reduction increases the surface area of the biomass and facilitates better heat transfer. General size reduction equipment used for agricultural residues are hammer mills, knife mills and tub grinders. The drying equipment includes perforated bin dryers, band conveyor dryers and rotary cascade dryers (Kumar et al. 2009).

There are many of parameters that play an important role in this process, namely design of a gasifier, gasification temperature, biomass flow rates, flow rates of oxidizing agent, type and amount of catalyst present and biomass properties (Kumar et al. 2009). The overall reaction of biomass gasification using air and/or steam can be represented by the equation 3.1 (Kumar et al. 2009). The formation of char and tar is usually because of the incomplete conversion of biomass (Kumar et al. 2009).

$$CH_xO_yN_zS_s \text{ (biomass)} + \text{air} + H_2O \text{ (steam)} \longleftrightarrow CH_4 + CO + H_2 + H_2O \text{ (unreacted steam)} + C \text{ (char)} + \text{ash} + \text{tar} \quad (3.1)$$

There are a many complex equilibrium reactions that occur in the solid, liquid and gaseous phase during biomass gasification (Huber et al. 2006). They are shown in Table 3.1. The ΔH value (Table 3.2) is the heat of the reaction; it is negative for exothermic reactions and positive for endothermic reactions. It can be observed that the oxidation reactions provide heat to the process and hence very little or no external heat needs to be

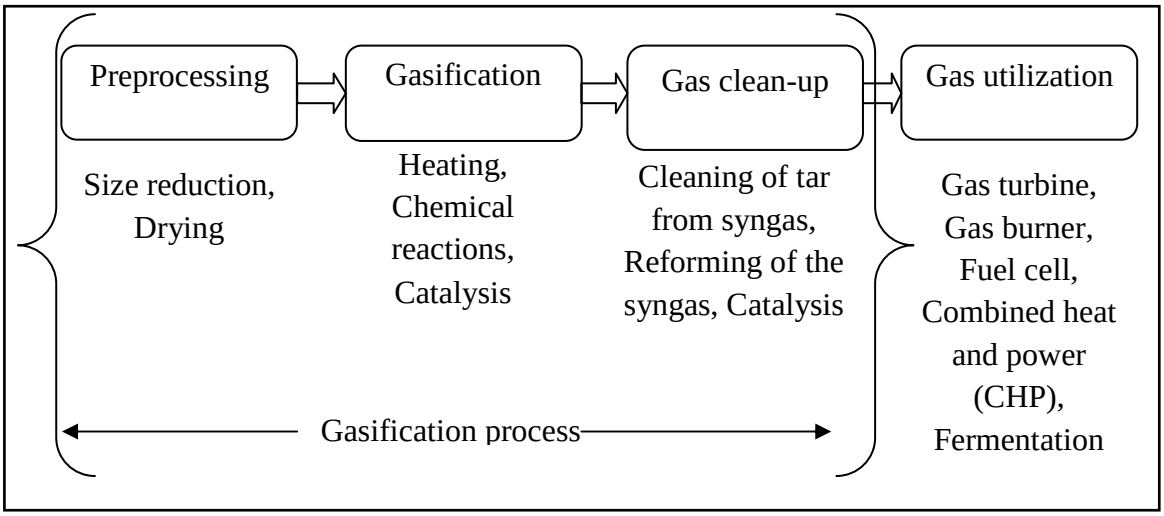

Figure 3.2 Overall flow diagram of a biomass- gasification process - Adapted from Kumar et al (2009).

Name of reaction	Reaction	ΔH (kJ/mol)	Equation number
Partial oxidation	$C + ½ O_2 \longleftrightarrow CO$	-268	(3.2)
Complete oxidation	$C + O_2 \longleftrightarrow CO_2$	-406	(3.3)
Methane formation	$CO + 3 H_2 \longleftrightarrow CH_4 + H_2O$	-206	(3.4)
Water gas shift	$CO + H_2O \longleftrightarrow CO_2 + H_2$	-42	(3.5)
Steam reforming	$CH_4 + H_2O \longleftrightarrow CO + 3 H_2$	-158	(3.6)
Water gas reaction	$C + H_2O \longleftrightarrow CO + H_2$	+118	(3.7)
Boudouard reaction	$C + CO_2 \longleftrightarrow 2 CO$	+165	(3.8)

Table 3.1 Common reactions during gasification (Kasteren et al. 2005; McKendry 2002b).

supplied (Belgiorno et al. 2003). Thus, based on the availability of oxidizing agents, gasification can be classified as direct or indirect gasification. Direct gasification is provided with an oxidizing agent (such as air or pure oxygen) and in indirect gasification, no oxidation agent is provided (Belgiorno et al. 2003; Huber et al. 2006). Equations 3.4 to 3.7 occur in the presence of steam during gasification. Besides stoichiometry, the product gas composition from a gasifier depends on biomass composition, gasification process and gasifying agent (Narvaez et al. 1996).

Gasifiers are classified based on the type of bed: fixed bed (updraft and downdraft) and fluidized bed. An updraft gasifier (Fig. 3.3a) is a counter flow set up where the biomass in introduced from the top and air is introduced from the bottom. It is named updraft as the product gas moves upward. Most of the combustion takes place in the bottom of the bed and this process produces a lot of tars (Klass 1998; Kumar et al. 2009; Reed 1981). On the other hand, downdraft gasifiers use concurrent flow. Both the biomass and the air are fed from the top and the producer gas is collected from the bottom part of the gasifier. Downward draft gasifiers (Fig. 3.3b) have more char and very low tars (Klass 1998; Kumar et al. 2009; Reed 1981). Fluidized bed reactors (Fig. 3.3c) consist of a fluidizing medium such as silica or alumina. The gasification agent (air, oxygen or steam) is allowed to pass through a bed of fluidizing medium, which at a certain velocity results in bed behaving like a fluid. The feed is introduced at the bottom with the gasification agent. These reactors have better conversion efficiencies, high heat transfer and uniform quality of the product gas and low levels of tars and char. The disadvantage of fluidized bed reactor is that they are more prone to attrition and poisoning (Cateni 2007; Klass 1998; Kumar et al. 2009; Reed 1981).

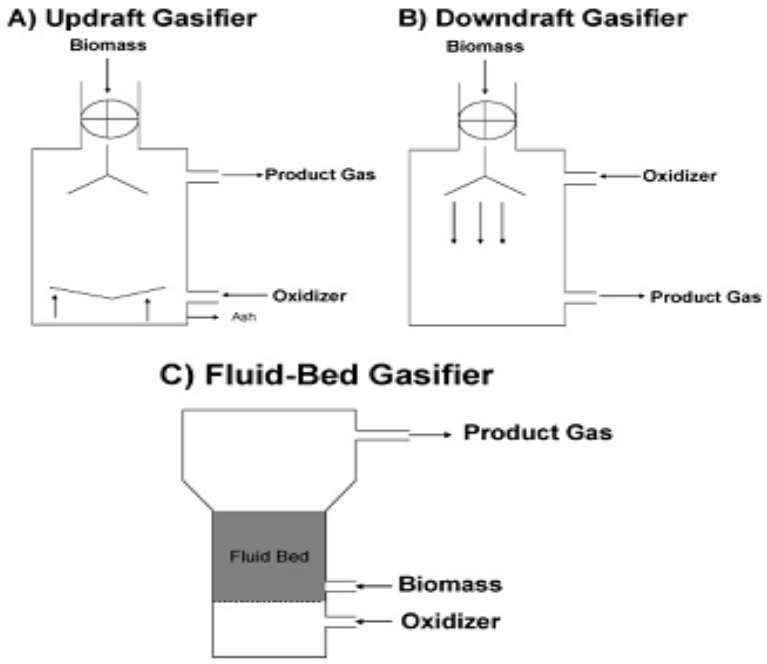

Figure 3.3 Gasification reactors - Adapted from (Huber et al. 2006).

Gas clean up and reforming (also called gas conditioning) is a major downstream process that is essential for effective utilization of producer gas by different processes (Huber et al. 2006; Kumar et al. 2009). The producer gas consists of many particulates, char, ash, tars, inorganic impurities (such as ammonia, hydrogen sulfide and many more) and hydrocarbons that can profoundly affect the gas utilization technology (Belgiorno et al. 2003; Bridgewater 1994; El-Rub et al. 2004). Among all these, tars has been the major problem in the scale up of processes (Kasteren et al. 2005). Tars can be destroyed at high temperatures, but generally the gasification temperature is below 1000°C (Huber et al. 2006; Kasteren et al. 2005). General methods of cleaning tars are wet scrubbing, dry scrubbing, dry-wet scrubbing and hot gas conditioning, catalytic conversion or a combination of these techniques (Huber et al. 2006). All these techniques involve reduction of tars or conversion of tars into CO and H_2, thereby, improving the overall yield of the gasification process (Bridgewater 1994). In some cases, reforming of syngas (process of changing the gas composition of a product gas to a desired composition) is done for a particular gas utilization process such as fuel cells applications (Kumar et al. 2009).

3.2 Fermentation process

The fermentation of producer gas is a more recently studied technology. It is carried out by acetogenic biological catalysts that convert producer gas into ethanol, acetic acid and biomass. The overall schematic diagram of the gasification-fermentation process is shown in Fig. 3.4. The conditioned gas from the gasifier is fed into a reactor that contains all necessary nutrients for anaerobic growth of the microbe. The microbe produces products such as acetic acid and ethanol, which could be recovered by distillation. There

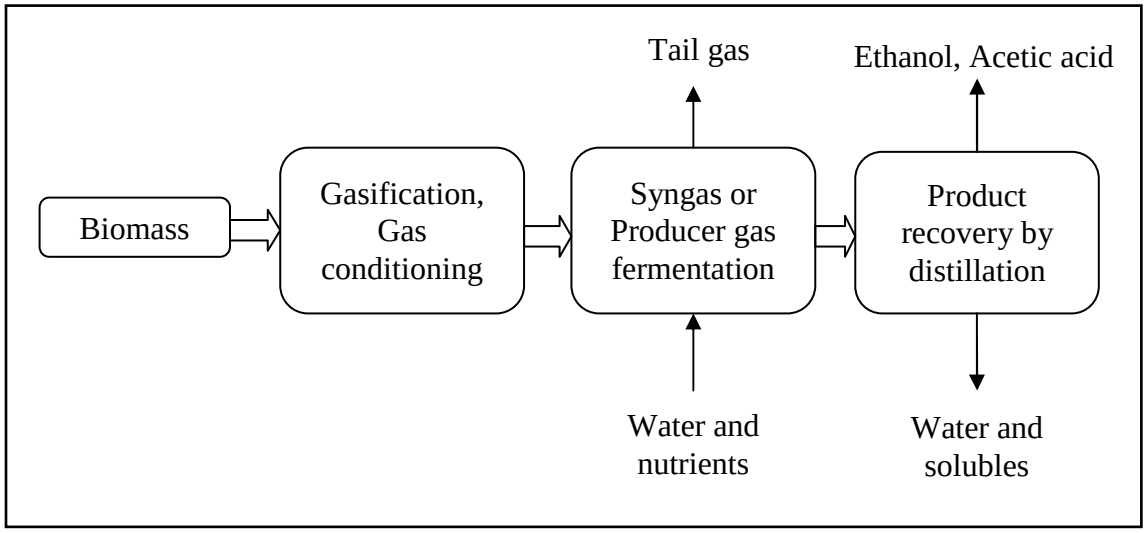

Figure 3.4 Block flow diagram of a gasification- fermentation process - Adapted from Spath and Dayton (2003).

are numerous advantages and several disadvantages of this process that are listed below.

Advantages:

1. Fermentation of producer gas by biological catalysts takes place at lower temperature and pressure than chemical catalytic processes, which drastically reduces the energy requirements thereby decreasing the operating costs. Because fermentations are carried out at atmospheric pressure, a specially designed reactor is unnecessary, which in turn will decrease the capital cost involved in the process (Heiskanen et al. 2007; Kasteren et al. 2005; Vega et al. 1988b; Worden et al. 1991).
2. Microbial processes have higher specificities, higher yields and better productivity, thus, the amount of by products is very low (Kasteren et al. 2005; Vega et al. 1988b; Worden et al. 1991).
3. Biological catalysts are not poisoned by trace contaminants like tars, hydrogen sulfide, sulfur dioxide and carbonyl sulfide that could reduce the cost of syngas clean up (Ahmed et al. 2006; Barik et al. 1988; Kasteren et al. 2005; Vega et al. 1990b; Worden et al. 1991)
4. Unlike other processes of syngas conversion, acetogens are very flexible with the CO/H_2 ratios and $CO/H_2/CO_2$ ratios (Huber et al. 2006; Kasteren et al. 2005). This would negate the use of gas-shift reactions (Heiskanen et al. 2007).
5. Gaseous substrates such as CO and H_2 allows uncoupling of hydraulic retention time with the supply of substrate that eventually would offer a better control of substrate and product inhibition (Henstra et al. 2007).

6. During anaerobic processes like syngas fermentation, the chemical energy in the gas is conserved in the products as no electron is lost to oxygen (Worden et al. 1991).
7. No hazardous or xenobiotic product is formed in the process (Worden et al. 1991).
8. The tail gas (unconsumed gas) is also rich in energy content, which can be either recycled or fed into another process.
9. The risk of contamination during producer gas fermentation is low because operating temperatures are either mesophilic or thermophilic, carbohydrate levels in the media are low, low operating pH and high CO levels that are inhibitory to many classes of microorganisms like methanogens (Spath and Dayton 2003).
10. Gasification- fermentation processes circumvent the problem of disposal of lignin (which are common in hydrolysis-fermentation process) as lignin can be gasified (Lewis et al. 2008).

Disadvantages:

1. The acetogens involved in bioconversions of syngas produce very little metabolic energy. This leads to slow growth and solvent production occurs only during non-growth phase (Tsai et al. 2009b).
2. Due to the slow reactions, the residence time and reactor volume is high (Vega et al. 1988b). In some cases, it may need special reactor design considerations (Barik et al. 1988).

3. Gas solubility is another major issue. Carbon monoxide and hydrogen in the gas must cross the gas-liquid interface and diffuse through the medium to reach the cell surface (Vega et al. 1988b).
4. The product stream is dilute, which increases the product recovery costs associated in the process (Vega et al. 1988b).

3.2.1 Stoichiometry of acetogenic bacteria

Bacterial conversion of CO, CO_2 and H_2 into ethanol, acetic acid and butanol takes place using the stoichiometric equations shown in Table 3.2. Also shown are the free energy of the reaction (ΔG^o), which is a thermodynamic measure of the possibility of a reaction. The reactions with highly negative free energies are more likely to occur. Formation of butanol and butyric acid is listed in this table as there are a few organisms such as *Clostridium carboxidivorans* and *Butyribacterium methylotrophicum*, that produce them (Datar 2003; Worden et al. 1991).

Rates of reaction(s) and yields of acids and/ or solvents depend on the type of species and/or strain, fermentation substrate, culture conditions and kind of products formed (Zeikus 1980). However, from the reactions listed above in Table 3.2, it is evident that formation of ethanol from CO is more favorable than acetic acid because the ΔG^o of the reaction 3.9 is greater than 3.13. But the ΔG^o of ethanol and acetic acid from CO_2 and H_2 (Eq. 3.10 and 3.14) are close to each other. Moreover, it can be observed that the formation of both acetic acid and ethanol from CO (Eq. 3.9 and 3.13) is more favorable than from CO_2 and H_2 (Eq. 3.10 and 3.14), clearly indicating that CO is a preferred substrate for carbon and energy. This supports the observations in our laboratory from gas analysis data of *Clostridium* strain P11 (that are presented in later chapters),

Reaction	ΔG^o (KJ/mole)	Equation number
$6CO + 3H_2O \rightarrow C_2H_5OH + 4CO_2$	-225	(3.9)
$6H_2 + 2CO_2 \rightarrow C_2H_5OH + 3H_2O$	-105	(3.10)
$2CO + 4H_2 \rightarrow C_2H_5OH + H_2O$	-137	(3.11)
$6CO + 6H_2 \rightarrow 2C_2H_5OH + 2CO_2$	-315	(3.9) + (3.10) = (3.12)
$4CO + 2H_2O \rightarrow CH_3COOH + 2CO_2$	-175	(3.13)
$4H_2 + 2CO_2 \rightarrow CH_3COOH + 2H_2O$	-95	(3.14)
$4CO + 4H_2 \rightarrow 2CH_3COOH$	-159	(3.13) + (3.14) = (3.15)
$10CO + 10H_2 \rightarrow 2C_2H_5OH + 2CH_3COOH + 2CO_2$	-570	(3.12) + (3.15) = (3.16)
$CH_3COOH + 2H_2 \rightarrow C_2H_5OH + H_2O$	-9.6	(3.17)
$10\ CO + 12H_2 \rightarrow 3C_2H_5OH + CH_3COOH + 2CO_2 + 2H_2O$	-824	(3.16) + (3.17) = (3.18)
$12CO + 5H_2O \rightarrow C_4H_9OH + 8CO_2$	-486	(3.19)
$12H_2 + 4CO_2 \rightarrow C_4H_9OH + 7H_2O$	NA	(3.20)
$12CO + 12H_2 \rightarrow 2C_4H_9OH + 4CO_2$	NA	(3.19) + (3.20) = (3.21)

Table 3.2 Stoichiometry of product formation from gaseous substrates; Adapted from (Barik et al. 1988; Phillips et al. 1994; Ragsdale 1991; Rajagopalan et al. 2002); NA- Not available.

Peptostreptococcus productus (Vega et al. 1988a) and *Clostridium carboxidivorans* (Shenkman 2003) that these microbes preferentially consume CO when a mixture of CO, CO_2 and H_2 is provided.

Different acetogens show preference to different gaseous substrates or mixture of gases for acid and/or solvent production. For example, *Acetoanaerobium noterae* can produce acetate, propionate and butyrate (Gaddy 1998). Other organisms such as *A. kivui*, *P. productus* and *Acetobacterium woodii* have shown the ability to carry over the reactions 3.13 and 3.14 (Gaddy 1998). *P. productus* has shown preference to equation 3.13 over 3.14 and demonstrates higher tolerance to CO (Vega et al. 1988a). However, *Clostridium ljundahlii* showed ability to carry out reactions 3.13 and 3.14 (acetic acid production) at faster rates that reactions 3.9 and 3.10 (ethanol production) (Gaddy 1998). Besides this, *C. ljundahlii* showed reactions 3.13 and 3.14 (production of acetic acid) at higher pH and reactions 3.9 and 3.10 (production of ethanol) at lower pH (Gaddy 1998). *C. carboxidivorans* shows ability to carry out ethanol production using equations 3.12 (with a yield of 0.33 moles of ethanol/ mole of CO consumed) and acetic acid production using 3.13 and 3.14 (Lewis et al. 2007). Moreover, Lewis et al. (2007) also observed that CO_2 was essential for the growth of this microorganism.

The yield of a fermentation process depends on the molar concentration of gases available (Datar et al. 2004). From the stoichiometric equations, it can be seen that one-third of the carbon in CO is converted into ethanol (Eq. 3.9), thereby, making theoretical conversion of CO to ethanol as 0.33 when CO is the only source of carbon (Rajagopalan et al. 2002). However, this theoretical conversion cannot be achieved practically because the acetyl-CoA pathway requires reducing equivalents which is provided from oxidation

of CO into CO_2 using the enzyme CODH (carbon monoxide dehydrogenase) (Datar et al. 2004). The fermentation process can take place in the absence of CO as well. When CO_2 and H_2 are available in the ratio 1:3, all the carbon in CO_2 can be converted into ethanol (through Eq. 3.10) (Datar et al. 2004). However, this stoichiometric molar concentration is difficult to achieve through gasification. Also, if we have an equimolar concentrations of CO and H_2, a theoretical yield of 0.667 can be achieved (sum of Eq. 3.12), or in other words, two thirds of the carbon in CO can be converted into ethanol (Datar et al. 2004). But, production of ethanol from CO and H_2 from equation 3.11 was also listed by Barik et al. (1988) and the possibility of its occurrence cannot be ruled out in *Clostridium* strain P11. However, acetic acid is an important growth related product in syngas fermentations. Thus, the actual overall equation of the fermentation process in the presence of CO, CO_2 and H_2 is shown in equation 3.16. According to this equation, four tenths of carbon in CO would go towards ethanol formation and four tenths would go towards acetic acid production, thus making the theoretical yield of both acetic acid and ethanol as 0.40.

3.2.2 Acetogens, their metabolism and energetics

Syngas conversion into acetic acid and ethanol is carried out by a special group of bacteria called acetogens. Acetogenic bacteria belong to one of the two metabolic groups of bacteria (other being methanogenic archae) that can grow autotrophically or can obtain virtually all of their carbon by reducing CO_2 (using acetyl-CoA pathway) with electrons derived from H_2 (Imkamp and Muller 2007; Ragsdale 1991). “Acetogens can be defined as obligately anaerobic bacteria that can use acetyl-CoA pathway as their predominant a) mechanism for reductive synthesis of acetyl-CoA from CO_2, b) terminal electron

accepting, energy conserving process and c) mechanism for the synthesis of cell carbon from CO_2" (Drake 1992). Acetogenic bacteria are one of the most versatile groups of organisms. They are found in a wide variety of habitats such as gastro-intestinal, terrestrial, subsurface and aquatic ecosystems and have ability to grow both chemoorganoheterotrophically (like sugars, C1 compounds, methoxylated aromatic compounds, acids and alcohols) and chemoautotrophically (on H_2 and CO_2) (Imkamp and Muller 2007).

The reduction of CO_2 for the formation of acetyl-CoA (the major metabolic intermediate in acetogens) occurs through the metabolic pathway called the acetyl-CoA pathway/ Wood pathway/ Wood-Ljundahl pathway (Ljundahl 1986; Ragsdale 1991). Acetogens cannot use the autotrophic Calvin cycle that is employed by many photosynthetic and chemosynthetic autotrophs as it lacks the enzyme ribulose diphosphate carboxylase (Rogers et al. 2006; Wood et al. 1986). Acetyl-CoA pathway is a non cyclic, irreversible pathway consisting of two reductive branches: the methyl branch and carbonyl branch as shown in Fig. 3.5 (Henstra et al. 2007; Ragsdale 1991). On the methyl branch, CO_2 is first reduced to formate using a NADP-dependant formate dehydrogenase enzyme. Formate is then converted into a formyl group bound to pterin hydrofolate with an expense of one ATP (energy). Formyl is further reduced (using four reducing equivalents/electrons) to a methyl group of a protein via methenyl, methylene and methyl intermediates via several tetrahydrofolate-dependant reactions (Drake and Kusel 2005; Henstra et al. 2007). There are numerous cofactors and enzymes that play an important role in the methyl branch. On the other hand, the carbonyl branch is dominated by only one enzyme: carbon monoxide dehydrogenase (CODH). This enzyme is also

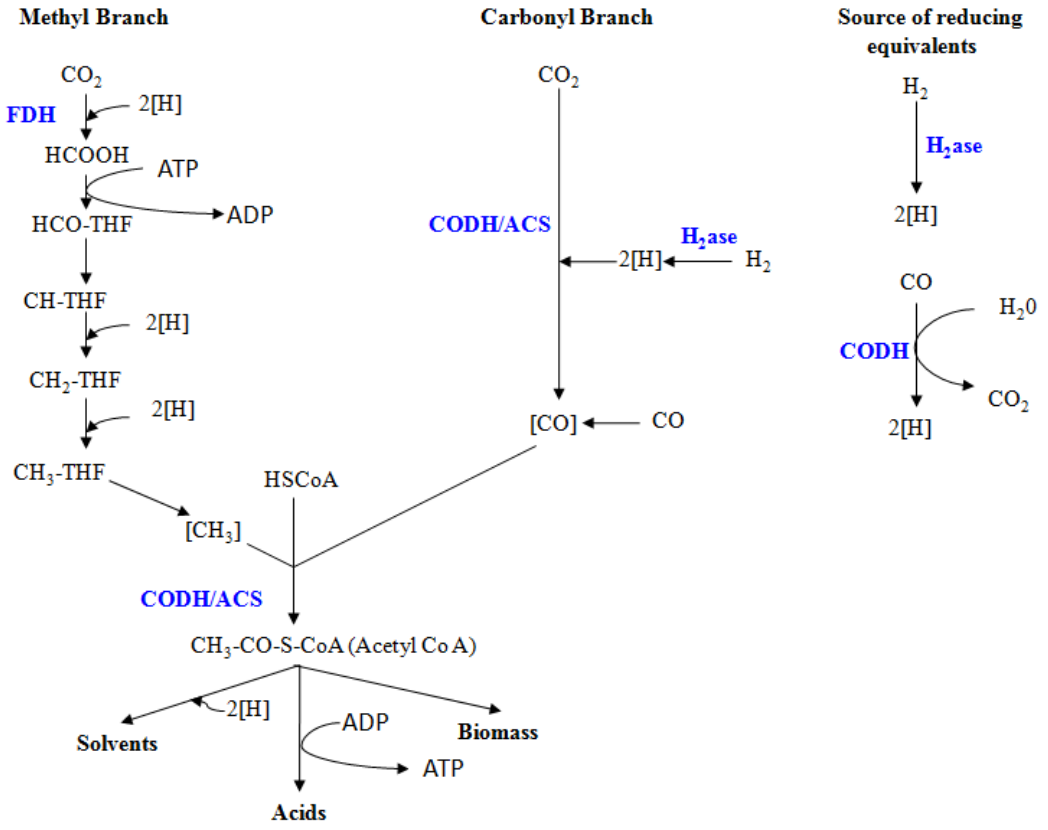

Figure 3.5 Overall picture acetyl-CoA pathway – Adapted from Henstra et al (2007); Enzymes involved in major steps are abbreviated, capitalized and shown in blue; FDH-Formate dehydrogenase; CODH/ACS- Carbon monoxide dehydrogenase/ Acetyl-CoA synthase; H_2ase-Hydrogenase.

called as CO: methylated corrinoid iron sulfur protein: CoA lyase or acetyl-CoA synthase (ACS) as it helps in the synthesis of the energy rich compound acetyl-CoA (Brock et al. 1994; Ljundahl 1986; Ragsdale 2004). If CO is readily available, it can be directly bound to the CODH-ACS enzyme. Otherwise, CO_2 is reduced to CO with the help of two reducing equivalents/electrons that then bind to the enzyme CODH-ACS. CODH and ACS is denoted as CODH/ACS to show their bi-functional character of both oxidation and reduction of CO and CO_2, respectively (Imkamp and Muller 2007). Finally, ACS assembles the two precursors (methyl and carbonyl moieties) with CoA to form acetyl-CoA. This energy rich molecule now serves as both a catabolic precursor (for acetate synthesis) and an anabolic precursor (for biomass synthesis) (Drake and Kusel 2005).

The formation of acetyl-CoA needs the investment of energy (Henstra et al. 2007). To recover the energy invested, an acetate molecule is formed by the enzymes phophotransacetylase and acetate kinase by the mechanism of substrate level phosphorylation (Henstra et al. 2007; Imkamp and Muller 2007). ATP is also obtained while producing other acids like butyric acid (shown in Fig. 3.6). The formation of solvents (like ethanol and butanol) requires reducing equivalents/ electrons as shown in Fig. 3.5. Fig. 3.6 describes the production of acids (acetic acid and butyric acid) and solvents (ethanol, butanol and isopropanol) in detail with the names of enzymes associated with the process. The production of butyric acid, butanol, acetone and isopropanol occurs when two molecules of acetyl-CoA are combined to form acetoacetyl-CoA. Conversion of acetoacetyl-CoA to butyryl-CoA requires more reducing power. Additional reducing power is need to produce solvents like isopropanol and butanol, but butyric acid production is associated with a release of ATP through substrate level

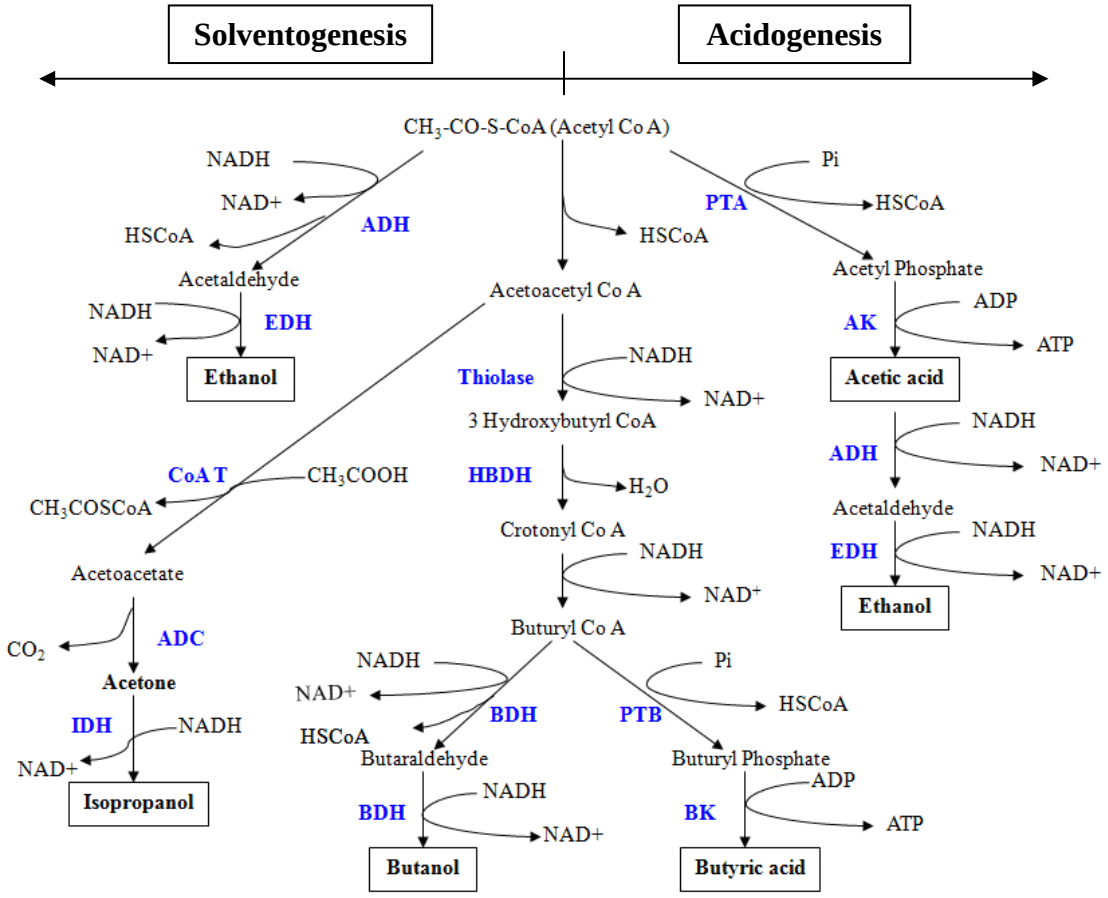

Figure 3.6 Production of different acids and alcohols from acetyl-CoA – Adapted from Brock et al (1994), Phillips et al (1994) and Vasconcelos et al (1994); Enzymes involved in major steps are abbreviated, capitalized and shown in blue; FDH- Formate dehydrogenase; CODH/ACS- Carbon monoxide dehydrogenase/ Acetyl-CoA synthase; H2ase- Hydrogenase; ADH- Acetaldehyde dehydrogenase; EDH- Ethanol dehydrogenase; PTA- Phosphotransacetylase; AK- Acetate kinase; CoAT- CoA-transferase; ADC- Acetoacetate decarboxylase; IDH- Isopropanol dehydrogenase; HBDH- 3-Hydroxybutyryl-CoA dehydrogenase; BDH- Butyraldehyde dehydrogenase and Butanol dehydrogenase in the formation of butyraldeyde and butanol respectively; PTB- Phosphotransbutyrylase; BK- Butyrate kinase.

phosphorylation.

The reducing equivalents required for both the branches of the acetyl-CoA pathway and/or for the production of solvents are obtained by either oxidation of hydrogen by the enzyme hydrogenase or by oxidation of CO by the enzyme CODH (These reactions are also shown in Fig. 3.5 under the title source of reducing equivalents) (Ragsdale 2004). Enzymes such as hydrogenase and CODH are located near the cytoplasmic membrane and play an important role in electron transfer mechanisms (Ljundahl 1986). Besides hydrogenase and CODH, other electron donors like NADH dehydrogenase and electron acceptors, such as methylene-H_4F reductase, are also associated with the cytoplasmic membrane (Imkamp and Muller 2007).

Besides substrate level phosphorylation, acetogens can conserve energy through the chemiosmotic mechanism (otherwise called electron transport phosphorylation) and in some conditions both processes can occur simultaneously (Drake et al. 2006; Imkamp and Muller 2007). Chemiosmotic mechanism involves generation of ATP through a transmembrane gradient using the enzyme F_1-F_0 ATP synthase (Imkamp and Muller 2007). It could be either proton (H+) dependant (as in *Moorella thermoautotrophicum*) or sodium (Na+) dependant (as in *A. woodii* and *Ruminococcus productus*) (Drake et al. 2006; Imkamp and Muller 2007). Besides these two systems, there are also sodium-proton antiporters (as present in *T. kivui*) that help to conserve energy in acetogens (Drake et al. 2006).

3.2.3 Biphasic fermentation pattern in solvent producing acetogens

Most acetogens such as *Clostridium acetobutylicum*, *Clostridium carboxidivorans* (formerly known as P7) and *Clostridium strain* P11 show a distinct pattern. They produce

acids (such as acetic acid and butyric acid) while in exponential growth phase, which leads to a decrease of pH from near neutral (around 6) to as low as 4 (Girbal et al. 1995b; Maddox et al. 2000). This phase of rapid decrease in pH and increase in acid concentrations is called acetogenesis (or acidogenesis). Formation of acids is growth related due to the concomitant production of ATP. The second phase, solventogenesis, is observed to be non-growth associated and leads to formation of reduced products such as ethanol, isopropanol and butanol; sometimes accompanied with a pH increase (Ahmed et al. 1988; Maddox et al. 2000).

The solventogenesis can be strongly influenced by the regulation of electron flow (Rao et al. 1987). This has been exhaustively studied on the microbe *C. acetobutylicum* for optimizing solvent yields. Studies on inducing solventogenesis in *C. acetobutylicum* and other microorganisms are discussed below:

1. *Increasing the partial pressure of CO*

It has been observed that CO inhibits clostridial hydrogenase, which plays an important role in hydrogen production to balance the excess reducing power generated by glycolysis (Girbal et al. 1995a). Thus, the electrons are directed towards reduction of NAD to NADH (instead of reducing ferredoxin) (Rao and Mutharasan 1986). Consequently, there is more reducing power available for solvent production which results in increase of butanol to acetone ratio (Bahl et al. 1986). However, this phenomenon of hydrogen production is not observed in *Clostridium* strain P11 as hydrogen is used as an electron donor in P11 fermentations.

2. Addition of reducing agents (or external electron mediators):

Many acetogens have a branched metabolism (as shown in Fig. 3.6) and the

product distribution changes with the redox potential (Mariotto et al. 1989). A number of reducing agents (like sodium thioglycolate, cysteine, ascorbic acid, sodium sulfide, titanium citrate, methyl viologen and others) have been added to fermentation media which has increased solvent formation (Mariotto et al. 1989; Rao et al. 1987). Rao and Mutharsan (1986) employed methyl viologen that decreased the hydrogen production and altered the electron flow towards NADH dependant alcohol formation in the microbe *C. acetobutylicum*. Besides *C. acetobutylicum*, positive effect on solventogenesis was also observed by the addition of methyl viologen on a strict anaerobic bacteria *Thermoanaerobacter ethanolicus* (Rao et al. 1987). Similar results were also obtained with benzyl viologen that showed the induction of solventogenesis at neutral pH contrary to the belief that solvent formation occurs only at low pH (Rao and Mutharasan 1987). Recent studies by Pannerselvam (2009) in our lab showed a two fold increase in ethanol concentrations when methyl viologen was used as a reducing agent in P11 fermentations when compared to cells that were not reduced with methyl viologen. Similar observations were also reported by Ahmed (2006) on *C. carboxidivorans* with neutral red as an electron mediator. There was an increase in ethanol, decrease in acetic acid and increase in forward alcohol dehydrogenase (ADH) activity, clearly indicating the regulation of electron and carbon flow towards ethanol formation.

3. Elevated ATP and NADH levels

ATP limitation by limiting glucose in continuous cultures enhanced acid production in *C. acetobutylicum* (Meyer and Papoutsakis 1988). Meyer and Papoutsakis (1988) experimentally showed that increase in ATP and NADH through CO gassing. Once ATP and NADH are readily available, the cells would produce reduced products

such as ethanol and butanol (Meyer and Papoutsakis 1988). Lower ATP demands and larger availability of reducing power leads to solvent production (Girbal et al. 1995b).

4. pH

pH is an important parameter in acetogens because it gives the first indication of the change in metabolism from acidogenesis to solventogenesis (Girbal et al. 1995b; Maddox et al. 2000). In batch cultures, solventogenesis has been correlated with pH and concentration of intracellular acids. Increasing the concentration of undissociated butyric acids by decreasing the intracellular pH increased acetone and butanol production in *C. acetobutylicum* (Monot et al. 1984). Higher intracellular acid concentrations was related to shift in metabolism of acetogens (Grupe and Gottschalk 1992) and the intracellular concentration of acids can be increased by addition of butyrate, propionate, valerate and 4-hydroxybutyrate at neutral pH (Jewell et al. 1986; Martin et al. 1983)

5. Nutrient limitation or addition

In general, the limitation of an essential nutrient responsible for the formation of a product that is undesirable (like acids) switches the mechanism towards solventogenesis. Junelles et al. (1988) showed that iron limitation affected the carbon and electron flow in the microbe *C. acetobutylicum*. The activity of hydrogenase decreased by 40% and the butanol-acetone ratio increased from 3.7 to 11.8. Decrease in the activity of hydrogenase was observed because iron is an important component of hydrogenase. Additionally, it has been reported that simultaneous addition of methyl viologen and depletion of iron from the media had an additive effect on butanol production using *C. acetobutylicum* (Peguin and Soucaille 1995). Contrary to these findings, a recent patent application by Lewis et al (2007) reported that an increase in ethanol production by a factor of two was

obtained when the concentration of iron was increased from 20 µM to 200 µM in the microbe *C. carboxidivorans*. This increase could be due to the reason that iron is an important constituent of the enzymes such as FDH, CODH and hydrogenase (Andreesen and Ljungdahl 1973; Drennan et al. 2004; Ragsdale et al. 1983; Vignais et al. 2001; Yamamoto et al. 1983).

Bahl et al. (1986) found that the butanol to acetate ratio increased by 1.9 times in a low phosphate synthetic medium with co-fermentation of lactate using *C. acetobutylicum*. The butanol to acetone ratio further increased from 2:1 to 8:1 in a media with low phosphate and iron limitation.

Yeast extract acts as a nitrogen source in syngas fermentations. Klasson et al. (1992) decreased the yeast extract concentration from 2 g/l to 0.05 g/l, which increased the ethanol-acetate ratio by two times. Another study showed that yeast extract was necessary for autotrophic growth (H_2, CO_2) of the microbes *Clostridium* strain F5a15, *Streptococcus* strain S5a2 and *Ruminococcus* strain S5a33; the biomass increased with the increasing concentrations of yeast extract (Leclerc et al. 1998). They found out that the vitamins in yeast extract played a crucial role in acetate synthesis. Barik et al. (1988) have mentioned that a 300% increase in ethanol to acetate ratio was obtained using a *Clostridium* species when yeast extract was completely removed from the media. Ammonium (nitrogen source) limitation studies conducted by Roos et al. (1985) on the microbe *C. acetobutylicum* also showed increased concentration of butanol with lower ammonium-glucose ratio in a pH uncontrolled fermentation.

Other important components of media for acetogens are trace metals, minerals and vitamin solution (Wiegel et al. 2006). A report from Bioengineering Resources Inc.

(Anonymous 1995) describes that the limitation of trace metals and minerals had little effect on ethanol/acetate ratio in the microbe *C. ljundahlii*. However, decreasing the concentrations of B-vitamins such as biotin and thiamine stimulated both growth and ethanol concentrations and eliminated the production of acetic acid. Contrarily, acetic acid concentrations were increased by decreasing the concentrations of calcium pantothenate (Anonymous 1995).

Glycerol is a more reduced substrate than glucose. *C. acetobutylicum* cultures grown on a mixture of glucose and glycerol had a seven fold increase in NADH and 2.5 fold increase in ATP concentrations when compared to cultures grown on glucose (Vasconcelos et al. 1994). They also observed decreased hydrogenase activity and increased alcohol dehydrogenase activity, thus, leading to increased concentrations of ethanol and butanol and decreased concentrations of acetic acid, butyric acid and hydrogen.

3.2.4 Sporulation and degeneration in clostridia

Sporulation is a defense strategy developed in certain kind of bacteria (such as *Bacillus* and *Clostridium* species) to overcome unfavorable environmental conditions such as heat, nutrient limitation, loss of water, irradiation etc (Durre 2005). During unfavorable conditions, the metabolism of bacteria reduces to a minimum and numerous distinct morphological and cytological changes take place such as elongation of the cells, formation of cigar shaped structures and more (Durre 2005; Jones et al. 1982). Clostridia generally form endospores, but other structures like exospores and cysts have also been reported (Durre 2005). Jones et al. (1982) carried out an exhaustive study on solvent production and morphological changes in *C. acetobutylicum*. They found a positive

correlation between sporulation and solventogenesis. The cells showed granulose accumulation 1-2 hours prior to the pH breakpoint (a point where acidogenesis ends and solventogenesis is induce and within 1-2 hours of this point, 90% of the cells attained swollen, phase bright, gram positive clostridial form. The culture that was sporulating produced almost 56 times more ethanol than the non sporulating mutants. Moreover, these clostridial forms (swollen, phase-bright presporulation-stage cells were involved in further conversion of acetate and butyrate into acetone and butanol. An explanation for the increased solvent production comes from studies conducted on *Clostridium thermosaccharolyticum* that sporulation was associated with up-regulation of enzymes such as ethanol dehydrogenase and glucose-6-phosphate dehydrogenase that are important for ethanol production (Jones et al. 1982).

Sporulation genetics has been widely studied in *C. acetobutylicum* and *C. beijerinckii* (Rogers et al. 2006). It has been found that solventogenesis and sporulation are activated by a common regulatory element (Spo0A protein) and thus all the cells starting to form solvents will also form spores. Furthermore, Spo0A protein, a regulatory protein responsible for the induction of sporulation formed by the expression of Spo0A genes, has been found to control the shift from acidogenesis to solventogenesis in the microbes *C. acetobutylicum* and *C. beijerinckii* (Dürre and Hollergschwandner 2004; Harris et al. 2002; Ravagnani et al. 2000). Besides sporulation, some reports also suggests the presence of heat shock proteins (hsp74) induced by heat stress are responsible for solvent production (Terracciano et al. 1988). Popoutsakis (2005) reported that over expression of heat shock proteins (GroESL) resulted in an increased production and tolerance of butanol in the microbe *C. acetobutylicum*.

Long et al. (1983) developed a defined media for sporulation of *C. acetobutylicum* P262 and five other strains. Although, *C. acetobutylicum* P262 showed sporulation and solvent production with the defined media, other strains produced 7.5-11 times lesser solvents. This clearly indicates that different microorganisms have different optimum conditions for spore formation and no generalizations can be made based on the result of one study.

Contrary to the finding of Jones et al. (1982) and Long et al. (1983), Tracy et al. (2008) found an inverse correlation between butanol production and sporulation in *C. acetobutylicum* ATCC 824. They observed that this strain carried out multiple levels of sporulation, but the amount of vegetative cells was directly proportional to butanol concentrations. It was proposed that the clostridial form cell precursor was responsible for solvent production rather than clostridial form cells. Advanced technology such as flow cytometry and fluorescence assisted cell-sorting techniques were used to precisely study the endospore formation.

The process of inducing sporulation has been a common practice for inoculum development. Sporulation can be induced by heat shock treatment. This process of heat shocking has widely been used to overcome the degeneration issue observed in many clostridial species such as *C. butylicum* (now called as *C. beijerinkii*), *C. pasteurianum*, *C. acetobutylicum* and others (Calam 1980; Gapes et al. 2000; Gapes et al. 1983; Kutzenok and Aschner 1952; Martin et al. 1983; Spivey 1978).

Clostridial strain degeneration is a widely observed, irreversible phenomenon by which solvent producing clostridia lose their ability to produce solvents when they are kept in vegetative state for a long time, which is a result of transferring actively growing

cells into fresh media (Kashket and Cao 1995; Rogers et al. 2006). For example, Gapes et al (1983) found that lactose utilization and butanol concentrations in *C. butylicum* increased for the first 3 subcultures, but after that degeneration was rapid. After the sixth subculture, the microbe did not produce any solvents. These observations are also noted in both repeated culturing of batch as well as continuous cultures (Finn and Nowrey 1959; Gapes et al. 1983; Kutzenok and Aschner 1952; Stephens et al. 1985). Strain degeneration is found to be a slow process and failure to induce solventogenesis is found to be associated with loss of important genes responsible for encoding the key enzymes for solvent production such as aldehyde/alcohol dehydrogenase, acetoacetate decarboxylase and acetoacetyl coenzyme A transferase (Stim-Herndon et al. 1996). Assobhei et al (1998) also observed an increase in enzymatic activities of acetate kinase and butyrate kinase that are responsible for the production of acetic acid and butyric acid in degenerated cells. Such culture degenerations have challenged the industrial production of solvents by the use of these microorganisms. To overcome this issue, cultures are maintained by repeated heat shocking (Kashket and Cao 1995).

The effect of heat shocking was carried on the microbe *C. beijerinkii* B592 over a wide range of temperatures (from 45 ºC to 95 ºC) and time (2.5 min to 10 min) (Gapes et al. 2000). They found that 95 ºC heat shocks for 2.5 minutes gave the highest butanol to acetone yield.

There are other observations that are very similar to degeneration phenomena noted during a particular fermentation run. These are called acid crash and acidogenic fermentation, which also leads to failure in the induction of solventogenesis. Previously, both these phenomena were confused with culture degeneration. Maddox et al. (2000)

defined acid crash as an early cessation of sugar uptake and solvent production when the culture pH is below 5 (for culture *C. beijerinkii* NRRLB592). This happened due to excess concentrations (about 57-60 mM or 3.6 g/l) of undissociated acids such as acetate and butyrate (Maddox et al. 2000). They suggested that acid crash could be prevented by having some pH control (to minimize the concentration of undissociated acids) or decreasing the metabolic rate of fermentation by decreasing temperature. On the other hand, acidogenic fermentation is characterized by high sugar utilization leading to fast growth and high acid production (total concentrations of 240-250 mM), but slow solvent production when the pH is controlled near neutrality and yeast extract is present in large amounts (Maddox et al. 2000). This phenomena can be prevented and solvent production can be regained by slowing down the glucose uptake rate or acid production rate by increasing the initial glucose concentrations and lowering the yeast extract concentrations (Maddox et al. 2000).

3.2.5 Effect of syngas contaminants in the fermentation process

Besides CO, CO_2, H_2 and N_2, producer gas has numerous contaminants like char, ash, tars (benzene, toluene, xylene and many more), inorganic impurities (such as ammonia, hydrogen sulfide, nitric oxide etc) and hydrocarbons (acetylene, ethylene, and methane) that can profoundly affect the scale up processes in fermentation process (Ahmed 2006; Belgiorno et al. 2003; Bridgewater 1994; El-Rub et al. 2004). Most of the studies to date in the area of gasification-fermentation employ the use of simulated or synthetic gas mixes (Girbal et al. 1995a; Tsai et al. 2009b). There is a belief that microbial catalysts can tolerate sulfides (such as carbonyl sulfide, hydrogen sulfide and sulfur dioxide), chlorine compounds and tars, but the effect of contaminants has never

been studied exhaustively (Barik et al. 1988; Spath and Dayton 2003). The cost of the overall process can be reduced drastically if the microorganisms are found to tolerate the contaminants (Spath and Dayton 2003). The limited literature on effect of contaminants have both shown positive and negative effects of syngas contaminants on growth and product distribution of a microbial catalyst. Most of the contaminants affect by the metabolism of the microbe by deactivating the enzymes such as CODH, hydrogenase and others.

Sulfur compounds such as hydrogen sulfide (H_2S), carbonyl sulfide (Gerhardt et al.) and sulfur dioxide (SO_2) are usually found in gas from coal gasification and they adversely affect chemical catalysts (Anonymous 1995). A report from Bioengineering Resources Inc. (BRI) shows that the presence of H_2S up to 2.5 % did not affect the uptake of CO and H_2 uptake rate and the growth of *C. ljundahlii* (Anonymous 1995). However, a strong inhibition to growth and gas uptake rate were observed when the concentrations were increased to 10%. However, the culture acclimated to sulfur gases showed an improved tolerance up to 20% (Smith et al. 1991; Vega et al. 1990b). Another CO utilizing microbe, *Rhodospirillum rubrum* was also found to degrade 5% carbonyl sulfide (Gerhardt et al.) within 20 h (Smith et al. 1991).

Effect of other contaminants like nitric oxide (NO) and acetylene (C_2H_2) has been studied on closely related species like nitrogen fixing bacteria and methanogens. Tibelius and Knowles (1984) observed the inhibition of oxygen dependant hydrogenase in a nitrogen fixing microorganism with nitrite, NO, CO, C_2H_2. Of the four, NO and nitrite was found to inhibit the hydrogenase irreversibly, but CO and C_2H_2 was found to have a reversible effect. Nitric oxide is formed due to some combustion effects during

gasification (West et al. 2005) and is known to be an inhibitor of hydrogenase (Hyman and Arp 1988; Krasna and Rittenberg 1954; Tibelius and Knowles 1984). It was found that nitric oxide deactivated hydrogenase activity in *Proteus vulgaris* at concentrations above 1%, but the inhibition was reversible at lower concentrations (Hyman and Arp 1988; Krasna and Rittenberg 1954). A slow, time dependant, reversible inhibition in the presence of NO was found in the NAD linked hydrogenases (in the microbe *Alcaligenes eutrophus*) which are responsible for oxidation of hydrogen (Hyman and Arp 1988). Tibelius and Knowles (1984) have also found NO to be a strong irreversible inhibitor to oxygen dependant hydrogenase. Reddy et al. (1983) suggested NO inhibited hydrogenase because it destroyed the four iron-sulfur centers which are important for the proper functioning of hydrogenases. Hyman and Arp (1991) explained the mechanism of reversible inhibition of NO in membrane associated hydrogenase in a nitrogen fixing bacteria, *Azobacter vinelandii*. They suggested that NO does not react at the nickel-hydrogen binding site. Rather, it is involved in interactions with iron sulfur centers which play crucial roles in enzyme catalysis and interaction. Effect of NO on *C. carboxidivorans* was studied closely by Ahmed and Lewis (2007). They observed that concentrations above 40 ppm NO acted as a non competitive, reversible inhibitor to hydrogenase. At the same time, they also observed increase in ethanol production by 5-7 times at all concentrations of NO. The reason for this increase was found due to increased activity of alcohol dehydrogenase enzyme.

Tars such as benzene, toluene, ethyl benzene, p-xylene, o- xylene and napthalene have been detected in producer gas produced from switchgrass gasification (Ahmed 2006; Ahmed et al. 2006). In batch experiments, it was observed that tars increased the

lag phase but also promoted ethanol concentration (by a factor of two) and decreased acetic acid concentration (by a factor of 2.5). The removal of tars using 0.025 µm filters prevented growth inhibition.

Besides tars, hydrocarbons such as methane (CH_4), acetylene (C_2H_2) , ethylene (C_2H_4) and ethane (C_2H_6) are generally found in biomass derived producer gases (Ahmed 2006). The concentrations of these contaminants are in the range of 1-7% CH_4, 0.1-0.5% C_2H_2, 0.5-2% C_2H_4 and 0.3-1% C_2H_6. C_2H_2 was found to be a slow binding, active site directed reversible inhibitor for nickel-ferrous hydrogenase present in nitrogen fixing bacteria that catalyze the oxidation of hydrogen (Sun et al. 2002). Ethane was found not to affect cell growth in *C. carboxidivorans* but acetylene and ethylene were found to increase cell mass by 33% and 55%, respectively (Ahmed 2006). The concentrations of products were not reported by Ahmed (2006), but it is definitely clear that cell growth was promoted by these contaminants. Although methane is believed to be an inert gas and has been used as a pressure indicator for gas analysis calculations (Vega et al. 1988a), effect of 4.5 % methane was carried out by Datar (2003) in *C. carboxidivorans*. He observed that concentrations of 4.5% methane did not affect gas utilization or growth, which confirmed the inert nature of methane to *C. carboxidivorans*.

It is difficult to make an atmosphere oxygen free during gasification, gas clean up and storage; quite often the presence of oxygen in producer gas has been reported (Datar 2003). Oxygen is one of the most toxic gases to acetogens because many of the enzymes present in the acetyl-CoA pathway are extremely sensitive to oxygen (Drake et al. 2006). However, there are some acetogens like *A. woodii*, *C. magnum*, *C. glycolicum* RD-1 and *M. thermoacetica* that can tolerate and consume oxygen at concentrations of 0.5%-6%

(Drake et al. 2006; Karnholz et al. 2002). *C. glycolicum* RD-1 is one the most interesting acetogens because it can withstand up to 6% oxygen (Kusel et al. 2001). It can simultaneously carry out acidogenesis and ethanol fermentation under anoxic conditions. In the presence of oxygen, it produces more ethanol, lactate and hydrogen. It is concluded that under oxic conditions, the metabolism of acetogens shift towards the catabolic pathway where the enzymes are less sensitive to oxygen (Drake et al. 2006; Kusel et al. 2001). Furthermore, a few acetogens such as *A. woodii, C. magnum, S. silvatica, M. thermoacetica, C. glycolicum* RD-1 contain enzymes involved in the removal of oxygen or its toxic products such as NADH-oxidase, peroxidsase, superoxide dismutase, rubredoxin oxidoreductase and rubrerythrin (Drake et al. 2006; Karnholz et al. 2002; Kusel et al. 2001). Datar (2003) studied the effect of oxygen on *C. carboxidivorans* and found that oxygen concentrations up to 1900 ppm (or 0.19%) did not affect CO and H_2 utilization, growth and product formation, which is indicative that *C. carboxidivorans* have mechanisms to remove the toxic products of oxygen.

3.2.6 Bioreactor designs and latest developments in producer gas fermentation technology

Gas liquid mass transfer is a major issue for syngas fermentations because of the low solubilities of H_2 and CO (Anonymous 1995; Klasson et al. 1992). The reaction rate (or gas transport rate), $dN_S{}^G/dt$ is given by (Anonymous 1995):

$$\frac{dN_S{}^G}{V_L dt} = \frac{K_L a}{H} P_S^G \tag{3.19}$$

Where, $dN_S{}^G$ is the number of moles of gas transported from the gas phase

V_L is the liquid volume of the reactor

t is time

K_La is the mass transfer coefficient

H is Henry's law constant

P_S^G is partial pressure of substrate in gas phase

From equation 3.19 it can be observed that the rate of reaction is proportional to the partial pressure of the gaseous substrates, which will in turn depend on the total pressure of the reactor (Anonymous 1995). Thus, increasing the pressure would increase gas solubilities (Henstra et al. 2007; Vega et al. 1990a). Also, from equation 3.19, the reaction rate is also proportional to the mass transfer coefficient of the gas, which will increase by providing large gas-liquid interfacial areas (Henstra et al. 2007; Vega et al. 1990a).

Continuous stirred tank reactors (CSTR) are the most widely used conventional reactors (Vega et al. 1990a). While using gaseous substrates, higher mass transfer coefficient (K_La) can be obtained by increasing the impeller speed (Henstra et al. 2007). Increasing the impeller speed breaks the larger bubbles into smaller bubbles. These smaller bubbles now have more surface area and lower rise velocities that eventually increase the gas-liquid contact time (Henstra et al. 2007). The disadvantage of using CSTRs is that the cost of the process increases drastically when the agitation speed is increased (Henstra et al. 2007). However this cost can be kept low by developing more efficient ways of sparging. A multi-orifice ring sparger (MORS) is one of the designs that can increase gas holdup distribution and reduce poorly mixed zones without increasing power input (Varma and Al-Dahhan 2007). Increasing the pressure in the CSTR could

also enhance the mass transfer coefficient, which in turn increases the reactor productivities (Vega et al. 1990a).

Bubble column reactors, on the other hand, provide large liquid retention times and/or large liquid hold up (Vega et al. 1990a). They can thus provide high interfacial area and high mass transfer coefficient with decreased cost associated in the process due to fewer moving parts and less maintenance (Charpentier 1981). Bubble column reactors are better for the use of fermentations using gaseous substrates than CSTRs because of these advantages and Vega et al. (1990a) showed that for a same retention time and mass transfer rate, bubble column reactors can give a 95% CO conversion while the CSTR can provide only 80% conversions.

However, the major challenge in producer gas fermentations is the need to increase cell yields in reactors. This can be achieved by cell recycle or cell retention (Tsai et al. 2009b). Cell retention could be achieved by fixing cells on inert solid support through adsorption, entrapment or covalent bond formation (Qureshi et al. 2005). Klasson et al. (1992) carried out immobilizing cells in a column reactor which resulted in very high cell densities and CO conversions. In fact, these conversions were better than bubble column and CSTR, which was mainly attributed to the fact that the operational parameters mimicked plug flow (Klasson et al. 1992). Cell retention can also be achieved through biofilm formation without the help of chemicals (Qureshi et al. 2005). This happens when the cells adhere naturally to an inert support over time. Cell densities as high as 74 g/l have been produced using this technique (Qureshi et al. 1988). Recently, Qureshi et al. (2005) reviewed the production of ethanol, butanol, lactic acid, acetic acid, succinic acid and fumaric acid production using biofilm reactors. But, these type of

reactors suffer from drawbacks such as low gas dissolution rates and very large reactor sizes (Tsai et al. 2009b).

Hydrophobic hollow fiber membrane (HFM) reactors can also improve mass transfer of producer gas to the biofilm (Khanal 2008). This technology offers an advantage of no loss of gas in the form of bubbles, which is commonly seen in CSTR and bubble column reactors (Khanal 2008). It also offers flexibility of controlling partial pressure of gas in the membrane lumen. Moreover, the surface area for efficient gas transfer can be changed without affecting other process parameters (Lee and Rittmann 2002). A hollow fiber bioreactor for nitrate removal from drinking water was developed was developed by Nerenberg and Rittman (2004). This HFM bioreactor allowed 100% transfer of H_2 to the biofilm for the reduction of nitrate into nitrogen as well as effective removal of other contaminants such as perchlorate, bromated, chlorate, chromate, selenate, selenite and dichloromethane. The major disadvantage of hydrophobic HFM reactors is that the gas transfer rates would be decreased if water condenses/deposits on the hydrophobic porous membrane (Tsai et al. 2009b).

The latest development in the area of producer gas fermentations is the development of hydrophilic asymmetric membrane bioreactors (Tsai et al. 2009a; Tsai et al. 2009b). Assymetric membranes are widely used in microfiltration and nanofiltration units. This set up has a gas contacting side and a liquid contacting side. The liquid contacting is supported in a porous spongy layer that promotes and controls the growth of microbes as it is in contact with nutrients for the microbes and removes the metabolic products of fermentation like ethanol, butanol, acetic acid and butyric acid. The gas is in direct contact with the microbes through the semipermeable micropores which maximizes

the gas utilization rates and dissolution rates up to 100% (Tsai et al. 2009c). The semipermeable micropores allow the transfer of gas into the liquid but do not allow the reverse. Concentrations of products as high as high as 6.4 g/l of ethanol, 4.8 g/l of butanol, 2.5 g/l of acetic acid and 1.5 g/l of butyric acid has been achieved using the hydrophilic asymmetric membranes using *C. carboxidivorans* in 20 days using a gas mix of 40% CO, 30% H_2 and 30% CO_2 (Tsai et al. 2009b).

Industrialization of gasification-fermentation technology will be possible by thoroughly understanding the metabolism of the acetogens, engineering the product yields and by employing bioreactor systems with efficient mass transfer of gases into liquids.

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CHAPTER 4

EFFECT OF BIOMASS GENERATED PRODUCER GAS AND ITS CONTAMINANTS IN FERMENTATIONS USING *CLOSTRIDIUM* STRAIN P11

4.1 Introduction

The need to reduce dependency on foreign oil coupled with other factors like global warming, political instability of oil producing countries and concerns over national security has led to research in the production of liquid fuels such as ethanol, biodiesel and butanol from renewable biomass (Ananymous 2009a; Lashof and Ahuja 1990; Rogers et al. 2006). Ethanol production from primary agricultural feedstocks, i.e. sugarcane and corn, is a well demonstrated technology in countries like Brazil and the USA. These feedstocks are primarily a source of food, which has given rise to many ethical questions for instance whether corn should be used for food or fuel and availability of land to grow corn (Anderson et al. 2008). While the conversion of corn to ethanol will continue in the near future, other technologies using cellulosic feedstocks, such as corn stover, wheat straw, barley straw, rice straw, switchgrass, wood waste, urban waste and others, are currently being developed (Ananymous 2009b).

Two biological conversion processes exist for the conversion of cellulose to ethanol, namely, hydrolysis-fermentation and syngas fermentation (Huber et al. 2006).

In hydrolysis-fermentation, the complex structure of the plant is broken down by a pretreatment followed by an acid or enzymatic hydrolysis to release sugars which are then converted into ethanol by yeast or bacteria (Olofsson et al. 2008). Besides being an uneconomical, multistep-multiconversion process, it also suffers from the major drawback of not utilizing 25-30 % of the plant material that is lignin (Tsai et al. 2009b). On the other hand, syngas fermentation is a two step process which combines gasification and fermentation. In the first step, cellulosic feedstocks can be gasified to produce a combination of carbon monoxide (CO), carbon dioxide (CO_2) and hydrogen (H_2) gas (with other gases such as nitrogen, methane, ammonia, hydrogen sulfide and other trace gases) that is called synthesis gas, producer gas or syngas. This gas can then be fermented by anaerobic microbes (also called acetogens), such as *Clostridium ljundahlii, Clostridium autoethanogenum, Butyribacterium methylotrophicum, Clostridium carboxidivorans* and *Clostridium* strain P11, to form ethanol, isopropanol, butanol, hexanol and specialty chemicals, such as acetic acid, butyric acid and hexanoic acid (Abrini et al. 1994; Grethlein et al. 1990). The gasification-fermentation process can utilize all the carbonaceous components of the biomass, which results in better conversion efficiency (McKendry 2002a). Furthermore, this process has the advantage of using different feedstocks (energy crops, agricultural wastes, industrial wastes and forest waste) depending on their availability and also using municipal waste, coal, natural gas and reformed gas, thus, making gasification-fermentation a flexible technology (Tsai et al. 2009a).

Some acetogens can convert gases such as CO, CO_2 and hydrogen H_2 into products such as ethanol, butanol, acetic acid and butyric acid using a non-cyclic,

irreversible, reductive pathway called the Wood-Ljungdahl pathway (Henstra et al. 2007; Ljundahl 1986; Ragsdale 1991). Different acetogens have preference to different gaseous substrates and produce a combination of products (Gaddy 1998; Lewis et al. 2007; Vega et al. 1988a). Most of the studies in the area of producer gas fermentation employ the use of simulated or synthetic gas mixes (Girbal et al. 1995a; Rajagopalan et al. 2002; Tsai et al. 2009b; Ungerman and Heindel 2008). Very few studies have used producer gas obtained from biomass gasification (Datar et al. 2004, Kundiyana et al. 2009). Research into integrating gasification and fermentation to enhance ethanol production is necessary to improve the economics of the process. One challenge in the utilization of producer gas using acetogens is that producer gas that is generated from gasifiers has numerous contaminants like char particles, ash particles, tars (benzene, toluene, xylene, etc), inorganic impurities (ammonia, hydrogen sulfide, sulfur dioxide, carbonyl sulfide, nitric oxide etc) and hydrocarbons (acetylene, ethylene, and methane), that can profoundly affect the fermentation process (Ahmed 2006; Ahmed and Lewis 2007; Anonymous 1995; Belgiorno et al. 2003; Bridgewater 1994; El-Rub et al. 2004; Smith et al. 1991).

The first research using contaminants was studied on *C. ljundahlii* by Bioengineering Resources Inc. (Anonymous 1995), which showed no inhibition of CO and H_2 uptake when concentrations of hydrogen sulfide (H_2S) were 2.5%, but strong inhibition of CO and H_2 uptake were observed at concentrations of 10%. However, *C. ljundahlii* culture adapted to sulfide gases showed tolerance up to 20% of sulfides (Smith et al. 1991; Vega et al. 1990b). Another CO utilizing microbe, *Rhodospirillum rubrum* was also found to degrade 5% carbonyl sulfide (Gerhardt et al.) within 20 h (Smith et al. 1991). Both *C. ljundahlii* and *R. rubrum* are CO and H_2 consuming acetogens similar to

Clostridium strain P11 that is under investigation in this research. Studies on sulfide gases (H_2S and COS) were more prevalent because coal gasification released 1-2% sulfide gases and these sulfides were toxic to chemical catalysts involved in the Fisher-Tropsch process (Vega et al. 1990b). Thus, *C. ljundahlii* and *R. rubrum* were found to be more tolerant of sulfides than chemical catalysts.

In a recent study, tars present in syngas were found to inhibit the growth of *C. carboxidivorans* for the first 8-10 days but, after this period of inactivity, the bacteria produced more ethanol (by a factor of 2 times) and less acetic acid (by a factor of 2.5 times) when compared to cells that were not exposed to producer gas containing tars (Ahmed 2006; Ahmed et al. 2006). Among the hydrocarbons, methane is usually the most abundant found at concentrations in the range of 1.85-5% (Datar 2003). Methane at 4.5% was not found to affect the metabolism of *C. carboxidivorans* (Datar 2003). Ethane also was found not to affect growth of *C. carboxidivorans*, but acetylene and ethylene were found to increase cell mass by 33% and 55%, respectively (Ahmed 2006). Nitric oxide increased ethanol concentrations by 5 to 7 times, increased the activity of alcohol dehydrogenase and acted as a non competitive, reversible inhibitor to hydrogenase above 40 ppm (Ahmed and Lewis 2007). Besides acetogens, the effect of NO has been tested on few microorganisms (such as nitrogen fixing bacteria and methanogens) and experimental results have shown both reversible and irreversible inhibition to a wide variety of hydrogenase enzymes in *Proteus vulgaris*, *Alcaligenes eutrophus* and *Azobacter vinelandii* by interacting with iron sulfur centers that play crucial roles in enzyme catalysis and interaction (Hyman and Arp 1988; Hyman and Arp 1991; Krasna and Rittenberg 1954; Tibelius and Knowles 1984).

This research uses a syngas utilizing acetogen, *Clostridium* strain P11 (hereafter referred as P11), to ferment producer gas produced by the gasification of Kanlow switch grass to form ethanol and acetic acid. The main objective of this research was to study the effects of biomass generated producer gas with acclimated and unacclimated P11 cells in a lab scale using 250 ml bottle reactors. In addition, a contaminant study with 5% methane in synthetic gas was also performed to observe its effects on P11 fermentations as methane was the biggest contaminant of biomass generated producer gas.

4.2 Materials and Methods

4.2.1 Biomass and producer gas

Biomass generated producer gas was obtained by gasification of switchgrass (*Panicum virgatum* var. Kanlow). A fluidized bed gasifier was used to gasify switchgrass. The gas was cleaned using two cyclone separators to remove particulates such as ash, char and fine particles. It was then was passed through a scrubbing system with a mixture of 20% acetone and 80% water (maintained at 0°C) to condense tar in the gas that escaped cyclone separation. Producer gas was then compressed and stored in 77 gallon storage tanks at 860 KPa (125 psia). Nine gallon transportation tanks were filled using downward displacement of water and are stored at the laboratory at 586 KPa (85 psia) where bottle reactor studies were carried out. In this study, the producer gas generated from biomass was compared to a bottled gas mix which had a composition of 20% CO, 15% CO_2, 5% H_2 and remaining 60% N_2 (Superior Specialty Gas Inc., Tulsa, OK). This composition was used because the producer gas generated by the fluidized bed gasifier using switchgrass as a feedstock had these compositions in the past (Datar et al. 2004). For the contaminant study, 5% methane was included in the bottled gas mix by the

manufacturer, thus, the composition was 20% CO, 15% CO_2, 5% H_2, 5% CH_4 and remaining 55% N_2 and was compared to the control gas mix without methane (20% CO, 15% CO_2, 5% H_2 and remaining 60% N_2).

4.2.2 Microbial catalyst and culture medium

The microbial catalyst, *Clostridium* strain P11, was provided by Dr. Ralph Tanner, University of Oklahoma. This strain was originally isolated from a duck pond at the University of Oklahoma (Huhnke et al. 2006). For all the experiments, the bacteria was grown on a defined media containing per L: 30 ml of mineral stock solution (Table 4.1), 10 ml of trace metal solution (Table 4.2), 10 ml of vitamin stock solution (Table 4.3), 1 g of yeast extract, 10 g of N-morpholinoethanesulfonic acid (MES) buffer, 10 ml of 4% cysteine sulfide solution and 1 ml of 0.1% resazurin solution. There are changes in the composition of the mineral and trace metal stock solutions (that are shown in Table 4.1 and 4.2) because of the advice from Dr. Tanner that addition or omission of some media components was good for higher solvent production in P11 fermentations. The media compositions were updated to be at par with our research collaborators. All the chemicals were purchased from Sigma Chemical Company, St. Louis, Missouri except the yeast extract that was purchased from Difco laboratories, Detroit, Michigan.

4.2.3 Preparation of the culture medium and batch studies

All the batch fermentation studies were performed in 250 ml serum bottles with 100 ml of culture media. The culture media was prepared by mixing all its components (mineral stock solution, trace metal solution, vitamin solution, MES, yeast extract and resazurin) in an appropriate amount of deionized water in a round bottomed flask. The pH of the culture medium was then adjusted to 6.1 using 2 N potassium hydroxide

Component	Amount (g/l) (for switchgrass syngas experiment)	Amount (g/l) (for methane experiments)
Ammonium chloride	100	100
Calcium chloride	4	4
Magnesium sulfate	20	20
Potassium chloride	10	10
Potassium phosphate monobasic	10	10
Sodium chloride	80	0

Table 4.1 Composition of stock mineral solutions. Sodium chloride was deleted in the methane experiment because of the advice from Dr. Ralph Tanner, University of Oklahoma that removal of sodium chloride increased ethanol production.

Component	Amount (g/l) (for switchgrass syngas experiment)	Amount (g/l) (for methane experiments)
Cobalt chloride	0.2	0.2
Cupric chloride	0	0
Ferrous ammonium sulfate	0.8	0.8
Manganese sulfate	0.8	1
Nickel chloride	0.2	0.2
Nitrilotriacetic acid	2	2
Sodium molybdate	0.02	0.02
Sodium selenate	0.1	0.1
Sodium tungstate	0.2	0.2
Zinc sulfate	1	1

Table 4.2 Composition of trace metal stock solution. A different composition of manganese sulfate was used for the methane experiments because of the advice from Dr. Ralph Tanner, University of Oklahoma as per the fermentation optimization studies carried by them.

Component	Amount (g/l) (for all experiments)
p-(4)-aminobenzoic acid	0.005
d-biotin	0.002
Calcium pantothenate	0.005
Folic acid	0.002
MESNA	0.01
Nicotinic acid	0.005
Pyridoxine	0.01
Riboflavin	0.005
Thiamine	0.005
Thioctic acid	0.005
Vitamin B_{12}	0.005

Table 4.3 Composition of vitamin stock solution

solution. The culture media was then made anaerobic by boiling it in a microwave and then briskly passing nitrogen to remove all the dissolved oxygen from the media. The flask was sealed with a rubber stopper and transferred to an anaerobic glove box (Coy Laboratory Products Inc., Grasslake, MI) where the media was dispensed into bottles and sealed. The bottles were then sterilized in an autoclave (Primus Sterilizer Co. Inc, Omaha, NE) at 121ºC for 20 min. After autoclaving, the serum bottles were left to cool to room temperature and 1 ml of 4% sterile cysteine sulfide solution was added to 100 ml of media in the serum bottle. The bottles were then purged with producer gas (synthetic or biomass based) depending on the experiment and inoculated with a 10 ml of inoculum containing actively growing cells. The actively growing cells were obtained by sub-culturing the cells twice. Each stage of subculturing is referred to as a passage. All inoculum transfers between passages were conducted when the microbes were in the mid-exponential phase. The bottle reactor in which the microbes grew faster (as determined by optical density measurements) was chosen for inoculum transfers into the next stage, as it was expected to have more healthy cells than the bottle reactor that grew slower. All inoculum transfers from one passage to another were conducted from the same reactors, thereby, minimizing the culture variation from reactor to reactor and from one treatment to another. The method of preparation and composition of the culture medium in subculture stages remained the same as that of the experimental procedure. After inoculation the batch reactors were kept in a walk in room which was maintained at 37ºC on an orbital shaker (Innova 2100, New Brunswick Scientific, Edison, NJ) at 150 rpm. Cell concentration, pH, product concentrations and producer gas concentrations were

analyzed at regular intervals. The overall sketch of the three experiments is shown in Fig. 4.1 and 4.9. Throughout the experiments, producer gas was fed at 24 h intervals.

4.2.4 Analytical methods

Samples from bottle reactors were collected at periodic intervals for measuring cell mass concentration, pH and product concentration. Gas samples were also collected to analyze the gas consumed by the microbe in a particular time interval.

Cell mass concentration: Cell mass concentration was measured in optical density (OD) units using a UV-visible spectrophotometer (Varian Inc., Palo Alto, CA) at a wavelength of 660 nm. Optical density was converted into cell mass concentration by the linear relationship determined by Panneerselvam (2009):

Dry cell weight, X (g/l) = 0.396 * Observed OD – 0.0521 (4.1)

pH: Culture pH was measured at periodic intervals of 24 h using a pH meter (Themo Orion, Beverly, MA). After measuring OD and pH, the fermentation samples were centrifuged at 12,000 rpm for 10 min with a benchtop microcentrifuge (Fischer Scientific, Pittsburg, PA). The supernatant was collected, filtered using 0.45 µm nylon membrane filters (VWR International, West Chester, PA) and frozen.

Product analysis: The fermentation broth was analyzed for ethanol, isopropanol and acetic acid using gas chromatography (GC) connected with a flame ionization detector (FID). Two GC systems were used in the study. For the first study using switchgrass producer gas, an Agilent 6890 N GC (Agilent Technologies, Wilmington, DE) was used with a Porapak QS packing 80/100 mesh column (Alltech Associates, Deerfield, IL). Helium was used as a carrier gas at a flow rate of 29.6 ml/min for the first 6 min, which was then increased to 42 ml/min at 1°C/min^2. The oven temperature was

maintained at 160 °C for the first 6 min, which was then increased to 220°C at a rate of 5°C/min. The injector and detector temperatures were held at 175°C and 250°C, respectively. The total run time for the analysis of a sample was 18 min. Methanol at 2.5 g/l was used as an internal standard. The GC was calibrated at 5 levels using known concentrations of compounds and Chemstation software (Agilent Technologies, Wilmington, DE) was used to quantify the concentration of compounds in the unknown samples.

The Porapak column described above had the disadvantage of lower efficiency to separate compounds. For example, compounds like acetaldehyde, acetone and isopropanol eluted close to each other and other compounds like butanol and butyric acid almost co-eluted. Although these were not primary products, this became a major concern when acetone was observed in fermentations using CGF. To solve this issue, another column was purchased from J&W Scientific named DB-FFAP column (Catalog NO. 100-2000) that was custom made for our purposes, and it was installed in the GC (Agilent 6890 N GC, Agilent Technologies, Wilmington, DE). The capillary column was 25 m long with a film thickness of 0.33 µm. Hydrogen was used as a carrier gas at a flow rate of 2.3 ml/min for 1.5 min and then ramped at till 4 ml/min. Inlet temperature was 200°C and a split ratio of 50:1 was used. A FID was used at 250ºC with hydrogen and air at 40 ml/min and 450 ml/min. The oven was also ramped at multiple rates for the best separation. Initially, the oven was at 40°C for 1.5 minutes, after which the oven was ramped at 25ºC /min until 60ºC was reached and then ramped at 40 °C/min until the oven reached 235ºC. The total run time using this column was 10 min and it could separate the compounds like acetaldehyde, acetone, methanol, isopropanol, ethanol, butanol, hexanol,

acetic acid, propionic acid, isobutyric acid, butyric acid and hexanoic acid with a very high resolution. 2-butanol was used as an internal standard in this system at concentrations of 2 g/l. The GC was calibrated at 7 levels using known concentrations of compounds and Chemstation software (Agilent Technologies, Wilmington, DE) was used to quantify the concentration of compounds in the unknown samples.

Headspace gas analysis: The composition of headspace gases was measured by withdrawing 100 µl of gas samples manually in 100 µl sample lock gas tight syringes (Hamilton Company, Reno, Nevada) and injecting them in a Agilent 6890N GC system (Agilent Technologies, Wilmington, DE) which was equipped with a Carboxen-1010 PLOT (Porous layer open tubular) capillary column with a dimension of 30 m (length) X 320 µm (inner diameter) X 15 µm nominal diameter (Supelco, Bellefonte, PA). To ensure oxygen did not interfere with the sampling techniques, all gas samples were taken in an anaerobic glove box (Coy Laboratory Products Inc., Grasslake, MI). The GC inlet was run in a "split" mode with a split ratio of 30:1 (meaning 30 parts of the injected sample is vented and 1 part enters the column for sample detection) and at a temperature of 200°C. Argon gas was used as a carrier gas with initial flow rate of 0.4 ml/min for the first 12 min then ramped at 0.1 ml/min^2 until it reached 0.8 ml/min. The initial oven temperature was 32ºC for 12 min and was then increased at 30ºC/min until the oven reached 236°C and was held at that temperature for 1.2 min. A thermal conductivity detector (TCD) was used for detection and its temperature was maintained at 230°C. This method detected CO, CO_2, H_2, N_2, O_2, CH_4, C_2H_2, C_2H_4 and C_2H_6. TCD detected CO, CO_2, H_2 and CO_2 with great sensitivity but the sensitivity for hydrocarbons such as CH_4, C_2H_2, C_2H_4 and C_2H_6 was low. Thus, the sensitivity to hydrocarbons was increased by attaching TCD in

series with the FID. TCD is a non destructive detector, thus, all the compounds passing through TCD will reach the FID for detection. By connecting the TCD and the FID the sensitivity for hydrocarbons was increased by 5-10 times. The total run time for analyzing a gas sample was 25 minutes. The GC was calibrated at 6 levels using known concentrations of gas compounds. Different volumes of gases were injected to calibrate the GC and the calibrations were checked by injecting different known gas compositions. Chemstation software (Agilent Technologies, Wilmington, DE) was used to quantify the concentration of compounds in the unknown samples in mole percentage.

Nitric oxide determination: Nitric oxide (NO) was determined using a chemiluminescence analyzer (Sievers currently owned by GE Analytical Instruments, Boulder, CO). Concentration of NO in producer gas samples was calculated based on the calibration curves were made by injecting known concentrations of NO.

4.3 Results and discussion

4.3.1 Batch studies- Effect of switchgrass producer gas

Producer gas was generated using fluidized bed gasifier and switchgrass as feedstock. The producer gas compositions are listed in Table 4.4. The experimental outline is shown in Fig. 4.1. On the third passage, cells were subjected to switchgrass producer gas from initial time (t=0), as there was a necessity to explore if P11 cells could withstand the contaminants produced during gasification, and were compared to the control which were grown of synthetic gas mix. It was hypothesized that the fermentations in the presence of producer gas will have a lag phase similar to the observation made on *C. carboxidivorans* due to the presence of tars in producer gas

Gas Type	Producer gas composition (%)
Carbon monoxide (CO)	15.87
Carbon dioxide (CO_2)	14.22
Hydrogen (H_2)	6.33
Methane (CH_4)	2.32
Acetylene (C_2H_2)	0.12
Ethylene (C_2H_4)	0.53
Ethane (C_2H_6)	0.28
Nitric oxide (NO)	44.5 ppm
Oxygen (O_2)	0
Nitrogen (N_2) (Balance gas)	60.33

Table 4.4 Producer gas compositions from a fluidized bed reactor with switchgrass as feedstocks; The gas composition is the average of triplicate injections on Gas chromatography (GC) with a Thermal conductivity detector (TCD).

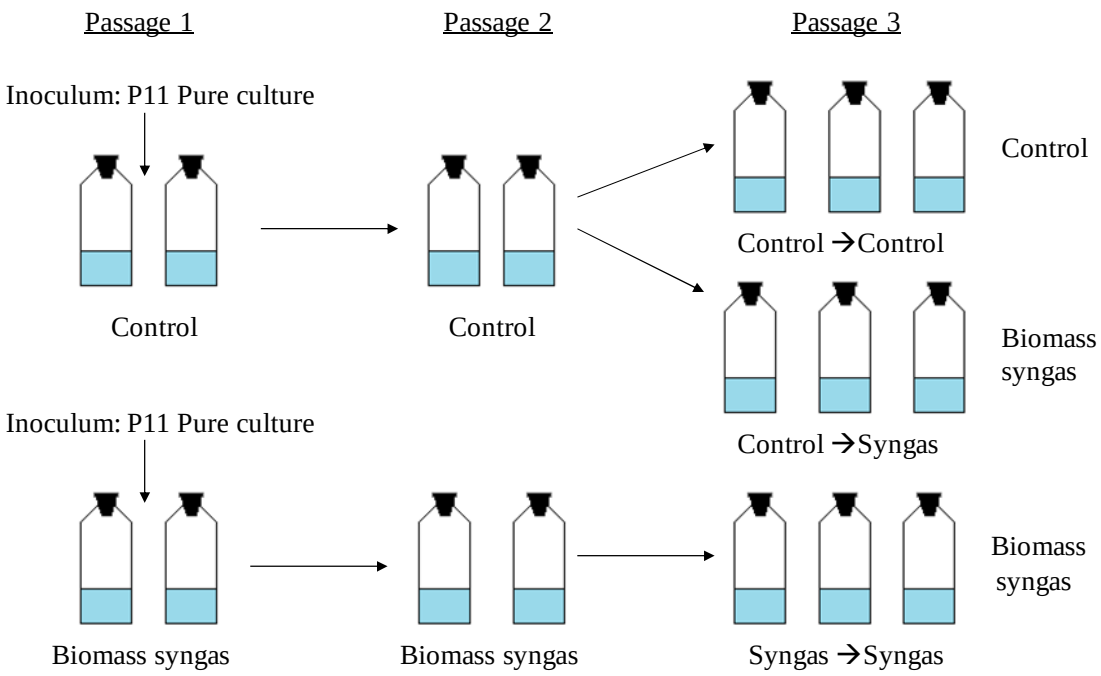

Figure 4.1 Experimental outline for switchgrass producer gas experiment; Control: Synthetic gas mix of composition 20% CO, 15% CO_2, 5% H_2 and 60% N_2 serves as substrate; Biomass syngas or syngas: Switchgrass producer gas of gas composition shown in Table 4.4 serves as substrate; Control → Control: Cultures grown on control gas mix throughout the experiment (Passage 1, 2 and 3). This serves as a control for the whole experiment; Control → Syngas: Cells that were grown in synthetic gas mix (in Passage 1 and 2) are transferred and grown on producer gas (in passage 3); Syngas→ Syngas: Cultures that were grown in producer gas throughout the experiment (Passage 1, 2 and 3) where cells are grown again on producer gas.

generated by the gasifiers (Ahmed et al. 2006). Another reason for using switchgrass generated producer gas from initial time (t=0) was that, industrial scale fermentations cannot afford the costly synthetic gas or simulated gas as it increases the operational cost of the process. Thus, the robustness of the microbe was tested by challenging it to grow on producer gas from initial time. Also, a parallel experiment was conducted by growing cells on producer gas during passaging to adapt the cells to the impurities of biomass generated producer gas. From the observations made from *C. carboxidivorans* fermentations (Ahmed 2006), it was hypothesized that the cells that are adapted to impurities would produce higher solvents than the cells which were not exposed to such an environment with impurities.

Cell growth and pH: Fig. 4.2 shows cell mass concentrations in different treatment levels. A vertical solid line shown in Fig. 4.2 at time 250 h is a demarcation line that will help understand the metabolic shifts during the fermentation that are explained during the discussion of results. At first, the two phases of cell growth can easily be noticed which are common to many fermentations, namely, growth phase for the first 200 h (approximately) and stationary phase after 200 h. Acetic acid was produced during the first 250 h (Fig. 4.5), after which ethanol was produced (Fig. 4.4). The two phases are called acidogenesis and solventogenesis respectively (Ahmed et al. 1988; Girbal et al. 1995b; Maddox et al. 2000). Acetic acid is growth associated because it is coupled with an energy (ATP) generation step (Henstra et al. 2007; Imkamp and Muller 2007). Media pH data (Fig. 4.3) was in accordance with the product data as acetic acid production was observed while pH decreased. After acidogenesis, the pH increased from 4.5 to 5.9 at the end of solventogenic phase. These observations of acidogenesis,

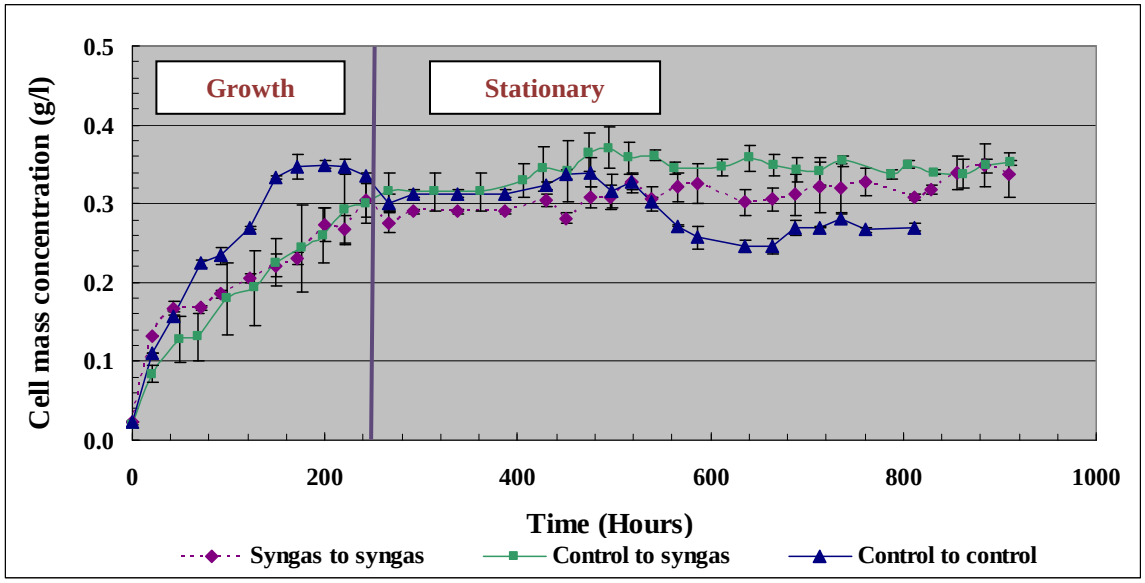

Figure 4.2 Cell growth in different treatments; Data shown is the average of number of replicates in each treatment; Error bars represent standard error; Number of replicates in the treatments Syngas to syngas, control to syngas and control are 3, 2 and 3, respectively. The treatment control to syngas has only 2 replicates because one of the bottle reactors accidentally broke during the start of the experiment.

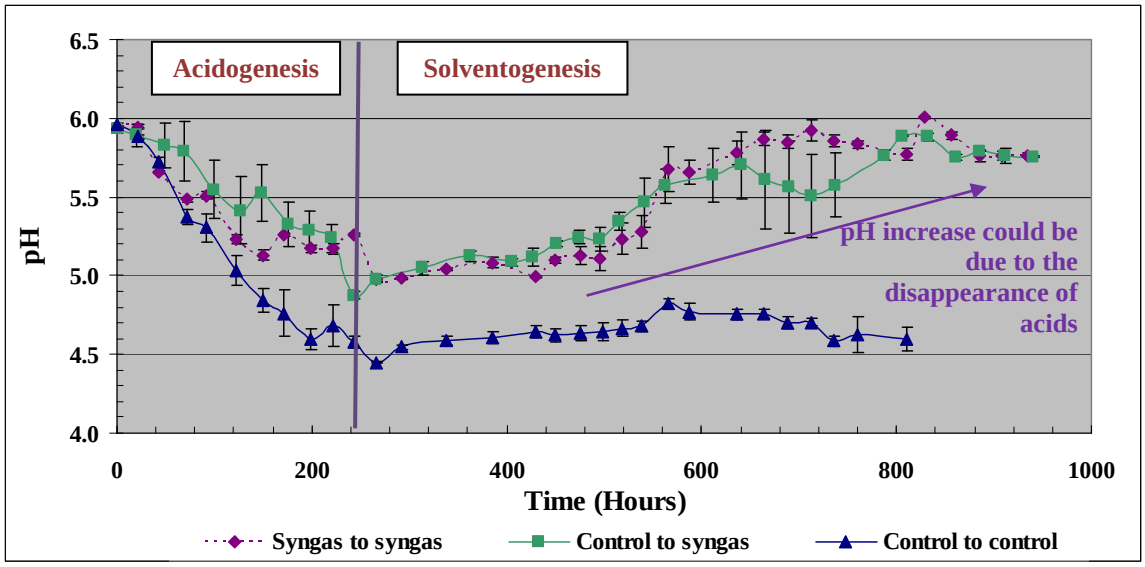

Figure 4.3 pH profile in different treatments. Data shown is the average of number of replicates in each treatment; Error bars represent standard error; Number of replicates in Syngas to syngas, control to syngas and control are 3, 2 and 3, respectively.

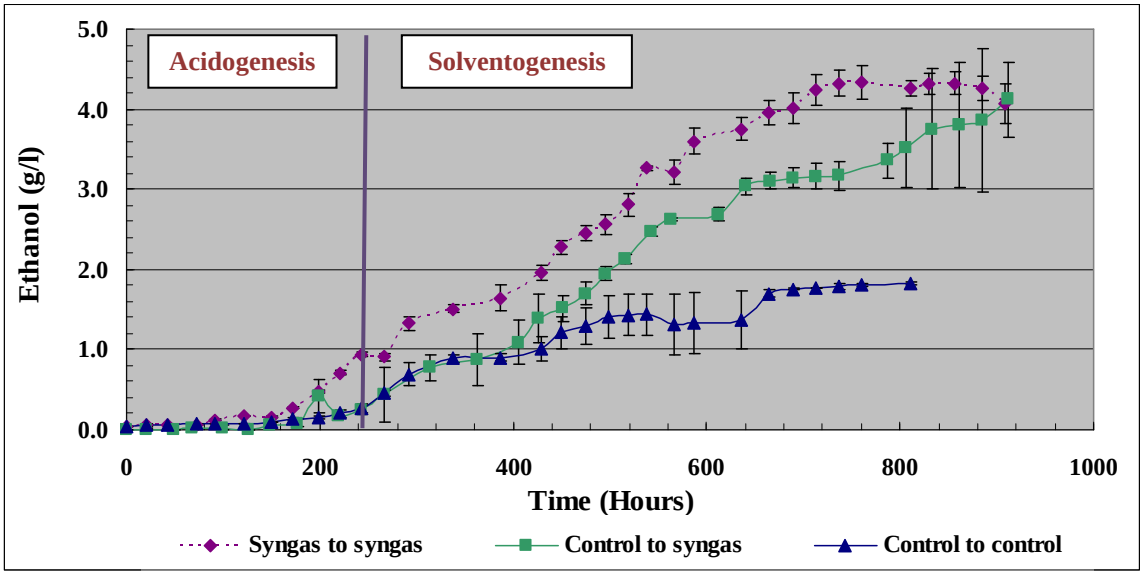

Figure 4.4 Ethanol production in different treatments. Data shown is the average of number of replicates in each treatment; Error bars represent standard error; Number of replicates in Syngas to syngas, control to syngas and control are 3, 2 and 3, respectively.

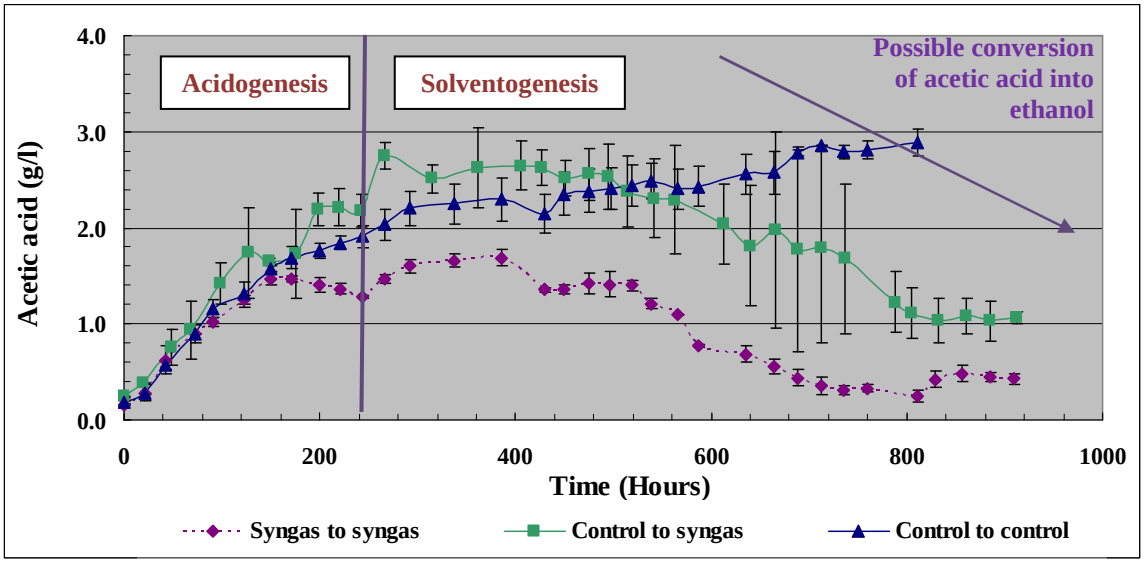

Figure 4.5 Acetic acid production in different treatments. Data shown is the average of number of replicates in each treatment; Error bars represent standard error; Number of replicates in Syngas to syngas, control to syngas and control are 3, 2 and 3, respectively

solventogenesis and pH shifts are in accordance with previous studies (Ahmed et al. 1988; Maddox et al. 2000). However, the pH of the control did not increase as much as the fermentations with producer gas. The reason for the pH increase could be due to the reduction of acetic acid into ethanol, which has been confirmed by research at University of Oklahoma by Dr. Ralph Tanner using C^{13} isotopes of acetic acid (Ralph Tanner, personal communication).

From Fig. 4.2, it can be observed that the control had the fastest growth as cell concentration reached 0.35 g/l in 170 h, whereas, the other treatments took longer to reach this cell mass concentration. This is because the cells were accustomed to the synthetic gas mix during passaging. The treatment in which cells were acclimated to switchgrass producer gas showed a faster rate of growth, during the first 50 h when compared to cells that were grown in synthetic gas mix and then transferred to switchgrass generated producer gas. This shows that P11 cells took some time to adjust to components in the producer gas, but over the full period of fermentation, there was not much delay to reach maximum cell densities. This is contrary to the observation by Ahmed et al. (2006) which showed a lag in growth for *C. carboxidivorans* for a period of 8-10 days when using producer gas. Furthermore, Ahmed et al. (2006) reported that this lag period was due to the presence of tars such as benzene, toluene, ethyl benzene, p-xylene, o- xylene and naphthalene. Besides the cyclone separator and acetone scrubbing, no further cleaning of producer gas was carried out and hence small concentrations of tars were expected in the producer gas. Although, the presence of tars in producer gas has to be confirmed but from the data on cell growth it can be easily noted that P11 cells may

have more tolerance to tars than *C. carboxidivorans*, thus making P11 a more robust organism in producer gas fermentations.

It was also observed that the control had a gradual decrease in cell mass concentration from 220 h until the end of the fermentation (Fig. 4.2). Unlike the control, the treatments which were exposed to switchgrass producer gas had steady cell mass concentrations. It can be observed that the pH increases and acetic acid concentrations decrease after 250 h. It could be possible that P11 has an alternative energy system for its energy conservation such as the chemiosmotic mechanism, otherwise called electron transport phosphorylation, which are common to some acetogens (Imkamp and Muller 2007). It is possible that the proton gradient which is formed due to the release of acetic acid helps the microbe with energy production (Fig. 4.6) for metabolic activities in the stationary phase as that does not allow the cell mass concentrations to decrease.

Product profiles: The products of P11 fermentations are primarily ethanol and acetic acid, however, isopropanol production was observed when switchgrass producer gas was used in this experiment. Ethanol, acetic acid and isopropanol production graphs are shown in Figs. 4.4, 4.5 and 4.7 respectively. There was an indication that some components of biomass producer gas not present in the synthetic gas mix were responsible for increased solvent production. When switchgrass producer gas was the substrate, a 123% increase in ethanol concentration was observed over when the synthetic gas mix was used (~ 4.1 g/l vs. 1.83 g/l). Also, isopropanol was produced, and acetic acid concentration was decreased by 41% when producer gas was the substrate as compared to the synthetic gas mix (1.69 g/l with switchgrass producer gas vs. 2.88 g/l with synthetic gas). The effect of compositional variation could have caused the difference in product

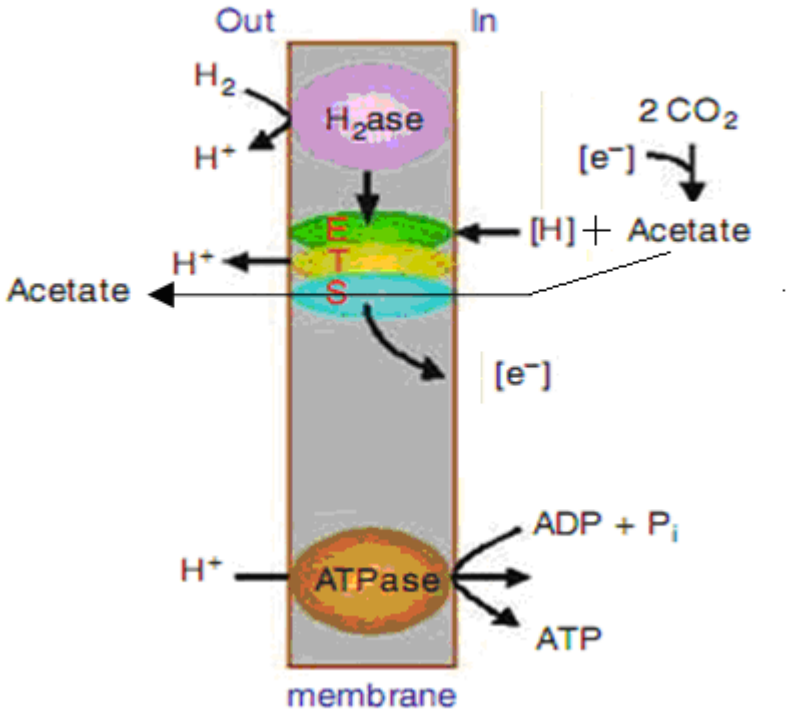

Figure 4.6 ATP generation from proton gradient; ETS is Electron Transport Chain; Hydrogenase adds proton outside the cells. Acetic acid produced inside the cells is excreted outside the cell developing more protons outside the cell. This proton gradient now generates ATP using membrane bound ATPase. Adapted from Drake et al. (2006).

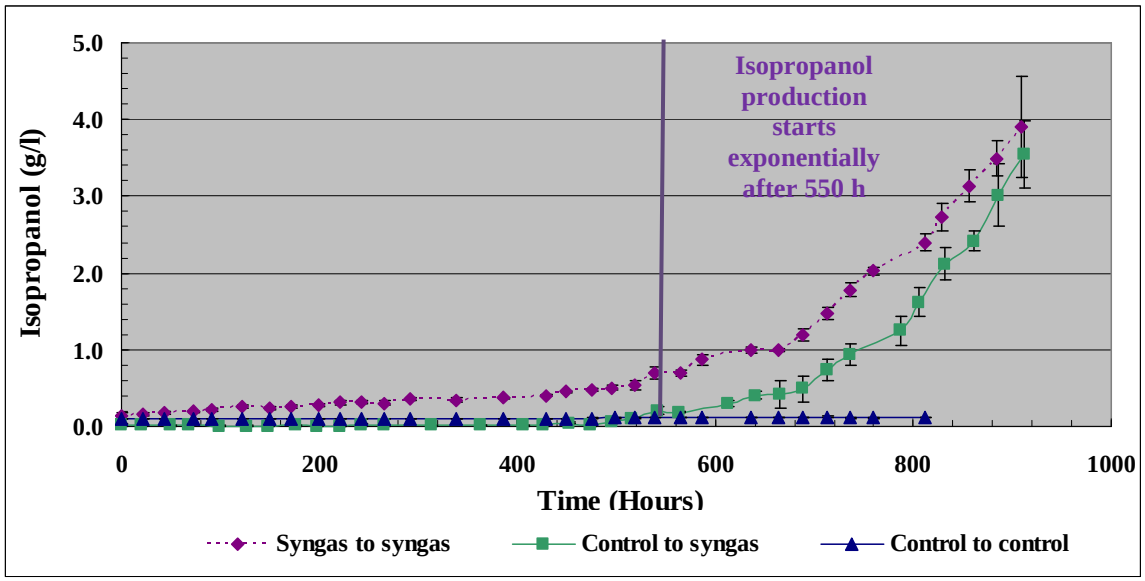

Figure 4.7 Isopropanol production in different treatments; Data shown is the average of number of replicates in each treatment; Error bars represent standard error; Number of replicates in Syngas to syngas, control to syngas and control are 3, 2 and 3 respectively.

concentration because CO, which is both a carbon and electron source, was 20.65% less in switchgrass generated producer gas (20% in synthetic gas mix and 15.87% in switchgrass generated producer gas) and H_2, which is an electron source, was 26.6% higher in switchgrass generated producer gas (5% in synthetic gas mix and 6.33% in switchgrass generated producer gas). If compositional variation had been the only reason for the observed metabolic shifts, then both adapted and unadapted cells should have performed similarly, with respect to product productivities. The exact effect of compositional changes of the gas needs to be determined by blending the gases with the help of mass flow controllers similar to composition achieved with producer gas generated from biomass by gasification.

Microbial growth requires enormous amounts of energy for synthesizing the building blocks of the microbe. Energy in the form of ATP is generated during acetic acid production by substrate level phosphorylation, and hence it is a growth related product. In all the treatments, acetic acid was observed as a growth related product, however, the amounts of acetic acid produced differed from one treatment to other (Fig. 4.5). In the first 250 h of fermentation, acetic acid concentration was found to be the highest with the treatment where cells were previously grown on synthetic gas mix and were transferred into next passage where switchgrass producer gas served as the substrate (Control to syngas). It is important to note that the number of cells in the treatment control to syngas was less than the control and hence acetic acid concentrations were compared based on g of acetic acid/ g of cell. It was observed that the treatment control to syngas had acetic acid yields 39.75% higher than the control (7.98 g of acetic acid/g of cells in control to syngas compared to 5.71 g acetic acid/g of cell in control). This indicates that

components of producer gas could be up-regulating the enzymes in the pathway for acetic acid production. Since the production of acetic acid for syngas to control is higher than the control, it can be deduced that the contaminants did not have any negative effect of acetic acid production, which demonstrates robustness of the microbe. The treatment syngas to syngas had 1.59 times lesser acetic acid than the control (1.69 g/l of acetic acid in the treatment syngas to syngas compared to 2.88 g/l in control). A culture which is adapted (or acclimated) to the contaminants of switchgrass producer gas showed that it could attain the same cell mass concentrations while producing less acetic acid (which is a growth related product). The energy derived for growth and metabolic activities could be coming from alternative ways, for instance, the energy conservation mechanisms in acetogens such as the chemiosmotic mechanism (Imkamp and Muller 2007). Since acetic acid is not the product of interest, reduced amounts of acetic acid is desired in the fermentations. Furthermore, the presence of biomass generated producer gas resulted in a decline in acetic acid concentration after 400 h with a concomitant increase in pH, increase in ethanol concentration and was associated with stable cell mass concentration. The standard error bars are larger during the decrease in acetic acid concentrations in the treatment control to syngas, which could be due to the culture heterogeneity as described in the past by Tracy et al. (2008) and Avery (2006). Also, it could be due to a lower number of replicates than the other treatments since one of the bottles was broken while conducting the experiments.

The theoretical conversion of acetic acid to ethanol can take place from the following reduction reaction:

$$CH_3COOH + 2H_2 \rightarrow C_2H_5OH + H_2O \quad (4.2)$$

Thus, the formation of ethanol from acetic acid can have a theoretical value of 0.77 g of ethanol/g of acetic acid (46 g of ethanol formed from 60 g of acetic acid). This raises the question of whether the observed higher amount of ethanol with switchgrass producer gas could be attributed to conversion of acetic acid into ethanol. To answer this question, an assumption was made that all the hydrogen responsible for the theoretical conversion of reaction 4.2 is available. This assumption was supported by some studies in our laboratory that showed consumption of CO during the stationary phases that could be a possible source of reducing equivalents necessary for the reduction of acetic acid to take place (data not shown). Calculations were made based on the theoretical amount of ethanol that can be produced from acetic acid to remove the effect of ethanol production from acetic acid in order to see if the producer gas contaminants enhanced ethanol from the acetyl-CoA pathway (Table 4.5). From the table, it could be deduced that acetic acid conversion to ethanol facilitated increase in ethanol concentrations (by 1.32 g/l in control to syngas and 1.11 g/l in syngas to syngas treatments). Despite the contribution of acetic acid, ethanol concentrations were still 53% and 63% higher in the treatments control to syngas and syngas to syngas than in the control, respectively (Table 4.5). This could be due to contaminants in producer gas that may have contributed in the up-regulation of the methyl and carbonyl branches of acetyl-CoA pathway (from CO and CO_2) to form ethanol.

Ethanol formation was induced earliest, 170 h, in the treatment syngas to syngas compared to 220 h in the control and control to syngas treatments (Fig. 4.4). The productivity of ethanol was also the highest in the treatment syngas to syngas (4 g/l was ethanol concentrations was achieved in 680 h) compared to control to syngas (909 h to

Treatment	Total ethanol (g/l)	Highest acetic acid (g/l)	Final acetic acid (g/l)	Total acetic acid consumed (g/l)	Theoretical ethanol from acetic acid (g/l)	Net ethanol not from acetic acid (i.e. from CO and H_2) (g/l)
Control to control	1.83	2.88	2.88	0	0	1.83
Control to syngas	4.12	2.75	1.03	1.72	1.32	2.8
Syngas to syngas	4.09	1.69	0.25	1.44	1.11	2.98

Table 4.5 Theoretical conversions of acetic acid to ethanol.

achieve 4 g/l). Thus, the culture that was adapted to producer gas had better solvent productivities.

Surprisingly, isopropanol production was observed with switchgrass producer gas at concentrations as high as 3.9 g/l (Fig. 4.7). While only 0.12 g/l of isopropanol was produced in the control, 3.55 g/l of isopropanol was produced in the treatment control to syngas and 3.9 g/l of isopropanol was produced in the treatment syngas to syngas. Isopropanol production was induced 100 h earlier with adapted culture (in the treatment syngas to syngas at 500 h) when compared to unadapted culture (treatment with control to syngas at 600 h). Exponential increase in isopropanol production occurred only after 550 h approximately, and during that time the rate of ethanol production slowed down (Fig. 4.4). It could be possible that enzymes responsible for ethanol production were inactivated due to the long experiment time, but the cells still had reducing energy (electrons) to produce isopropanol from acetone (Fig. 3.6). The production of isopropanol has been confirmed with producer gas fermentations carried by other colleagues in our group in pilot scale reactors (Kundiyana et al. 2009). Preliminary experiments in our laboratory have indicated that P11 has the ability to reduce acetone into isopropanol (data not shown). Further investigations are necessary to confirm the biological production of isopropanol in the presence of syngas, but this study showed that P11 has an enzyme that can reduce acetone to isopropanol.

The distribution of products for P11 is similar to observations made by Datar (2004) and Ahmed (2006) on *C. carboxidivorans*. Besides tars, nitric oxide (NO) was one of the contaminants that was found responsible for the metabolic shifts that increased ethanol concentrations by 5 to 7 times, increased the activity of alcohol dehydrogenase

and acted as a non competitive, reversible inhibitor to hydrogenase above 40 ppm (Ahmed and Lewis 2007). The concentration of NO in the switchgrass producer gas was found to be 44.5 ppm and it could be affecting the metabolic shifts in P11 similar to *C. carboxidivorans*. However, the effect of NO on P11 was not included in this study and needs to be researched further to understand the effect of NO on product formation in P11 fermentations. The treatment in which cells were adapted to syngas (syngas to syngas) had the highest mole percentages of solvents (ethanol and isopropanol = 94.5 mole %) and only 4.5 mole% acetic acid (Fig. 4.8). Treatment control to syngas equally performed well with 89.3 % solvents and 10.7% acetic acid while the control produced 46 % (mole) solvents and 54 % (mole) acetic acid. Molar percentages obtained with treatment syngas to syngas is desired at industrial scale although the concentrations of solvents were low for industrial production. Higher concentrations of ethanol (27.6 g/l) and isopropanol (9.3 g/l) have been reported by Kundiyana et al (2009) during a switchgrass producer gas fermentation over 45 days in a 75 L CSTR reactor while retaining the similar molar concentrations of solvent and acids (90.3% solvents and 9.7% acid). A deeper knowledge of the metabolism of the microbe and effect of individual components of producer gas will help favor higher concentrations of products with molar ratios similar to what has been achieved in bottle reactors. Also, the mass transfer of producer gas components, which is very low in bottle reactors, could be increased by using CSTR, bubble column and/or membrane reactors that will eventually increase the concentrations of ethanol (Kundiyana et al. 2009; Rajagopalan et al. 2002). This study has clearly showed that P11 produced more solvent using producer gas than using synthetic bottled gases with a similar CO and H_2 composition. Further

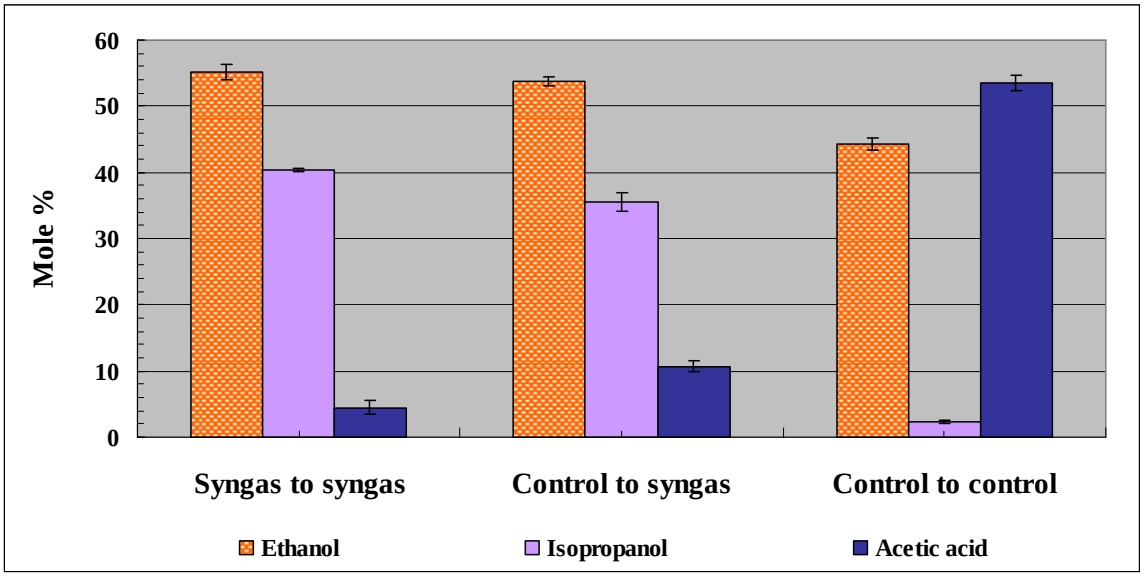

Figure 4.8 Mole percentage of products formed in different treatments. Error bars represent standard error.

investigations with the minor components of producer gas will help in understanding the role of these components in inducing metabolic shifts in producer gas fermentations.

4.3.2 Batch studies – Effect of 5% methane in P11 fermentations

Methane (CH_4), a major component of producer gas obtained by gasification of agricultural feedstocks was found to vary from 1.85% (in our studies) to as high as 5% (Datar et al. 2004). Although methane is considered as an inert component of producer gas in many studies using other microbes like *P. productus* (Vega et al. 1988a) and *C. carboxidivorans* (Datar 2003), it is still necessary to find the effect of methane because it constitutes the single largest hydrocarbon contaminant of producer gas. It was hypothesized that methane could have a major effect on producer gas fermentations as the experiment using switchgrass syngas showed a major product distribution. To test this hypothesis, an experiment was conducted with synthetic gas having 5% CH_4. The synthetic producer gas composition used to study the effect of methane was 20% CO, 15% CO_2, 5% H_2, 5% CH_4 and 55% N_2 whereas the control gas composition was 20% CO, 15% CO_2, 5% H_2 and 60% N_2. The overall experimental outline of this experiment is shown in Fig. 4.9. Two negative controls were also used in this experiment, one for each gas treatment. Negative controls were used to confirm whether apparent gas consumption was due to the microbe or by abiotic mechanisms such as the solubility of methane was in the media. Methane could be a potential contributor to the shift in metabolic activities observed in the switchgrass producer gas studies.

Growth and pH: The growth and pH profiles are shown in Figs. 4.10 and 4.11. In both treatments, exponential increase in growth was seen after a lag phase of 24 h which lasted from 24 h up to 70 h. The cells were in stationary phase until 200 h (in both

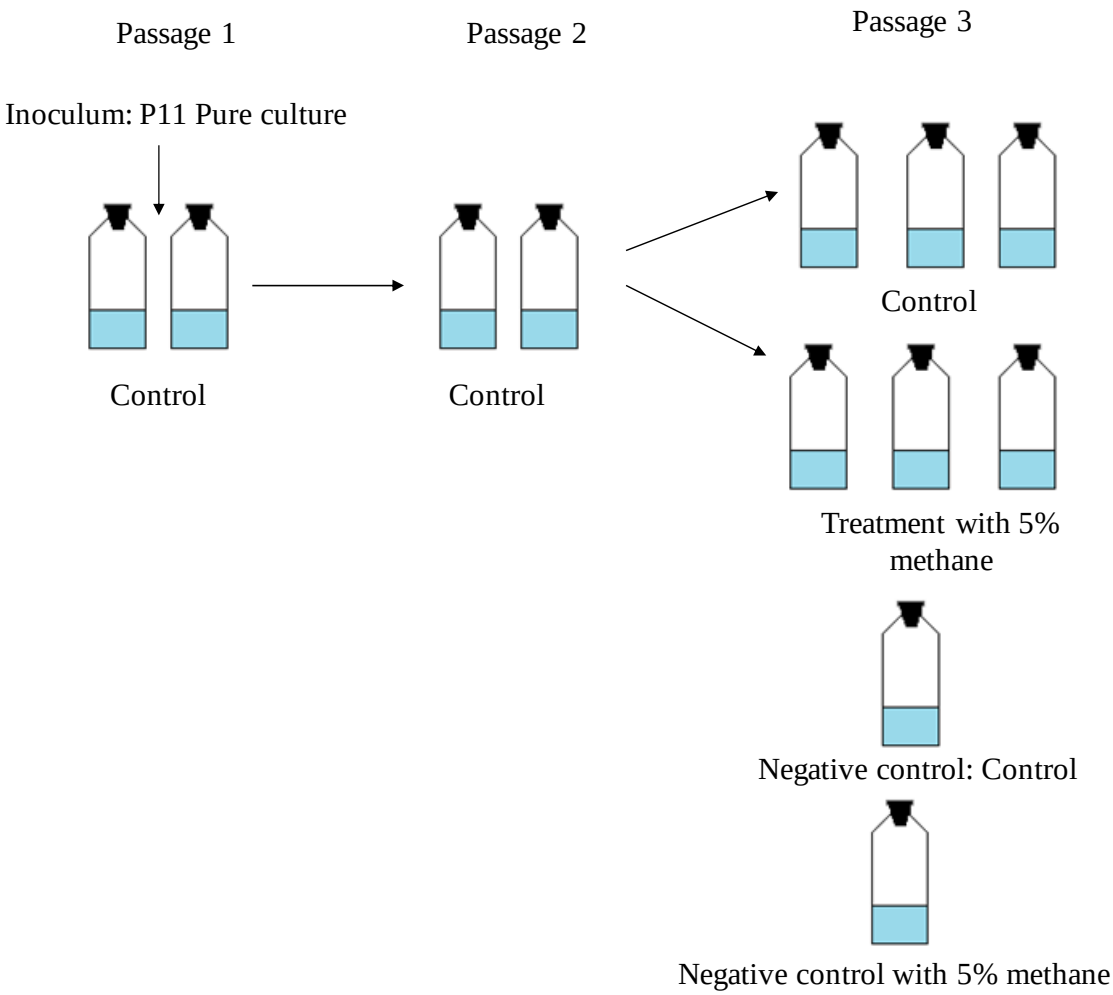

Figure 4.9 Experimental outline for contaminant experiment; Control-Synthetic gas mix of composition 20% CO, 15% CO_2, 5% H_2 and 60% N_2 as substrate; Treatment with 5% methane- Synthetic gas mix with 5% methane (Gas composition had a composition of 20% CO, 15% CO_2, 5% H_2, 5% CH_4 and 55% N_2); Negative control: Control- Uninoculated bottle reactor with synthetic (or control) gas; Negative control with 5% methane- Uninoculated bottle reactor with 5% methane included in the synthetic gas.

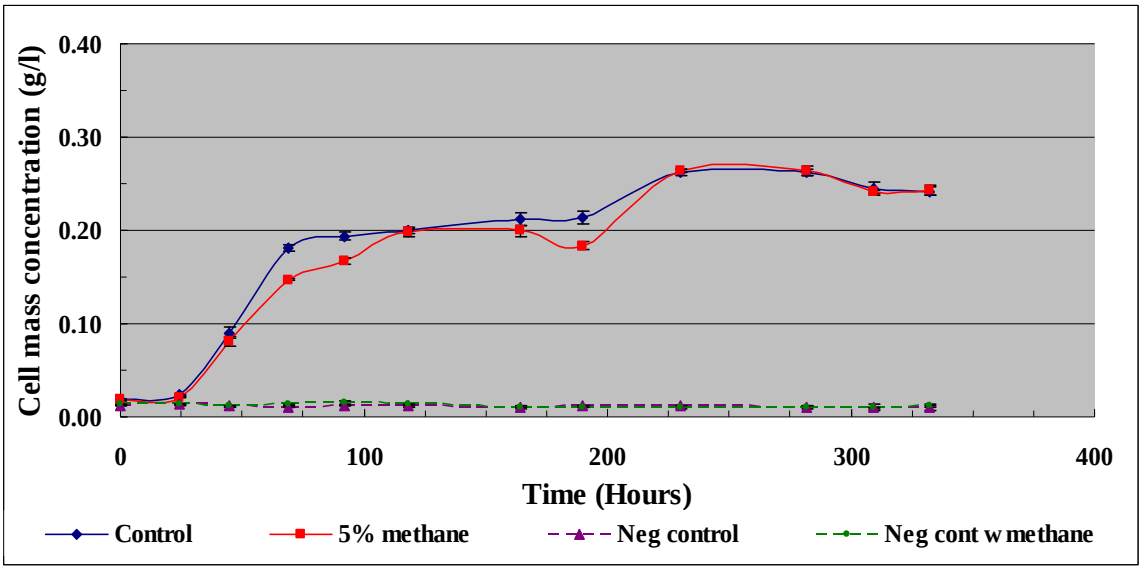

Figure 4.10 Cell growth in different treatments; Each data point is the average of 3 replicates except the negative controls where the number of replicates is 2; Error bars represent standard error.

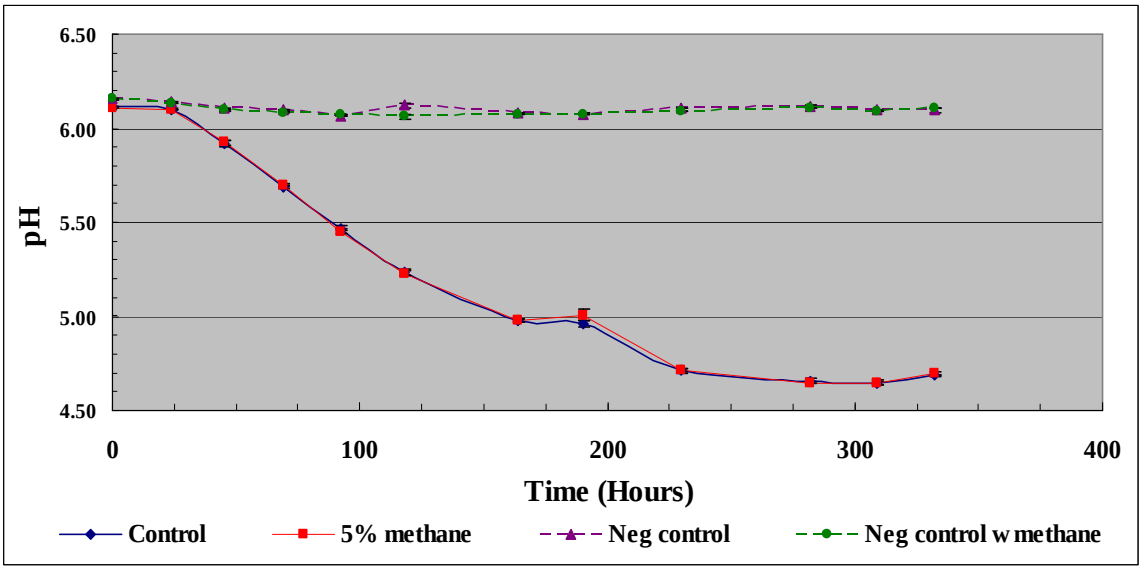

Figure 4.11 pH profiles in different treatments; Each data point is the average of 3 replicates except the negative controls where the number of replicates is 2; Error bars represent standard error.

treatments), which was followed by a secondary growth phase. The pH data is in accordance with the growth as there was a decrease in pH from 6.1 to 4.65 due to acetic acid production during the growth phase. The negative controls, showed no change in cell mass concentration and pH as expected. Similar cyclic behavior of growth and solvent production has been reported in *C. acetobutylicum* (Tracy et al. 2008). Other experiments with P11 have also shown similar behavior (data not shown). The growth and pH profiles in the presence of 5% methane are very similar to that of the control, which shows that 5% methane did not have any effect on P11 growth.

Product profiles: Ethanol and acetic acid production profiles are shown in Figs. 4.12 and 4.13. In the presence of 5% methane, ethanol production was only 15% higher (0.23 g/l in the presence of 5% methane compared to 0.2 g/l in control). This increase could be due to culture heterogeneity (Avery 2006; Tracy et al. 2008). Ethanol concentration at 190 h is unusually higher and could be an outlier. Also, only 6.93% variation between the control and 5% methane treatment in the acetic acid profiles (Fig. 4.13) indicate that methane did not affect the metabolic activities of the microbe which supports studies in the past. Vega et al. (1988a) used methane as a pressure indicator for gas analysis calculations in *P. productus*. Also, Datar (2003) observed that concentrations of 4.5% methane did not affect gas utilization or growth, which confirmed the inert nature of methane to *C. carboxidivorans*.

Gas consumption profiles: Consumption of the gaseous substrates CO, H_2, CO_2 and CH_4 are shown in Figs. 4.14, 4.15, 4.16 and 4.17, respectively. Gas analysis was performed only for 190 h of fermentation because of equipment failure. CO and H_2 consumption occurred throughout the growth phase of the fermentations and similar

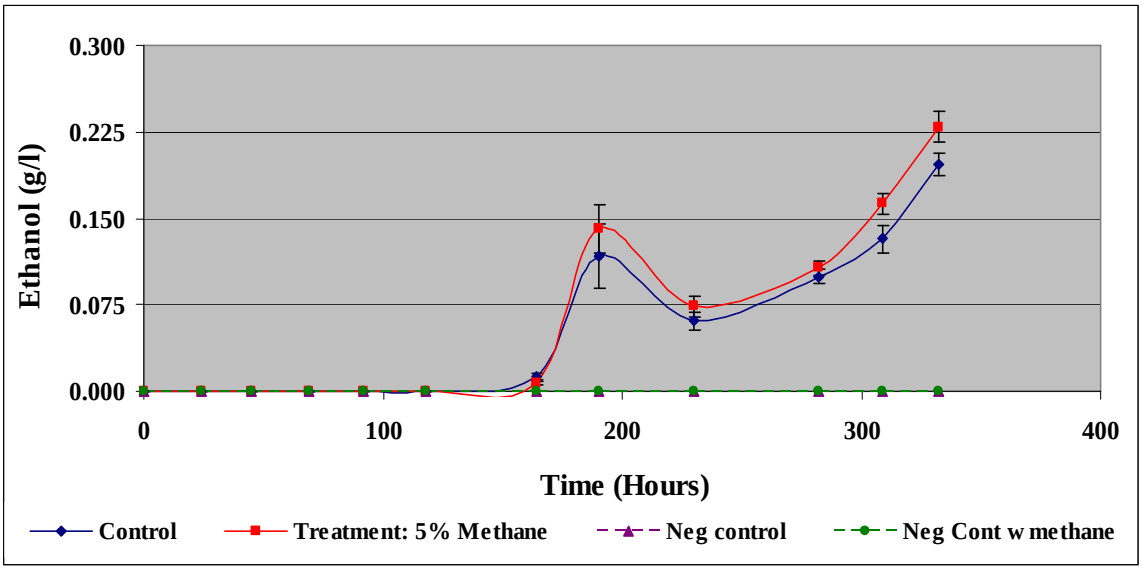

Figure 4.12 Ethanol production in different treatments; Each data point is the average of 3 replicates except the negative controls where the number of replicates is 2; Error bars represent standard error.

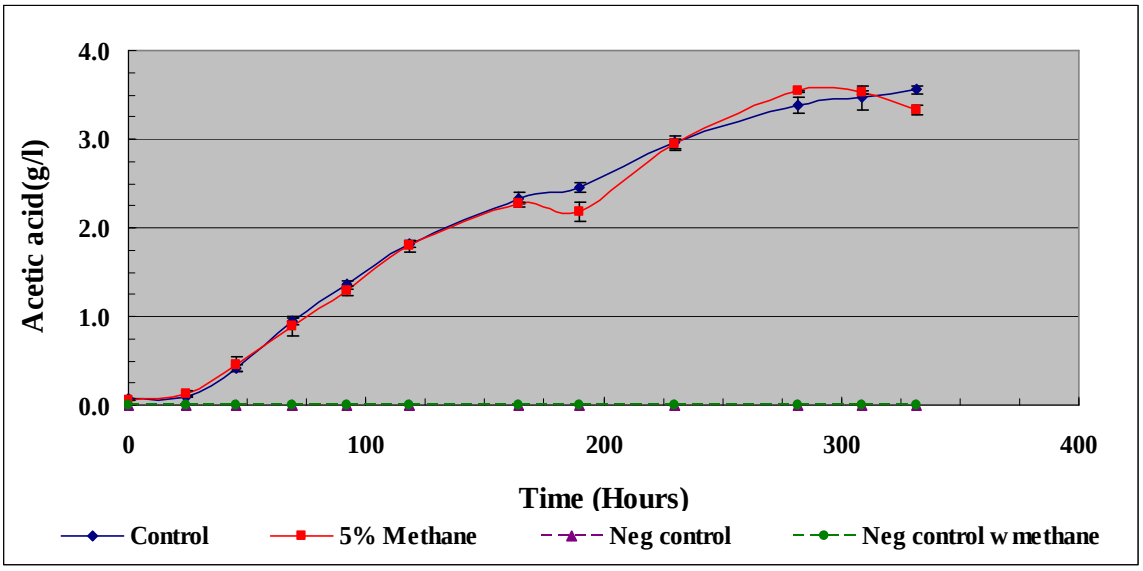

Figure 4.13 Acetic acid production in different treatments; Each data point is the average of 3 replicates except the negative controls where the number of replicates is 2; Error bars represent standard error.

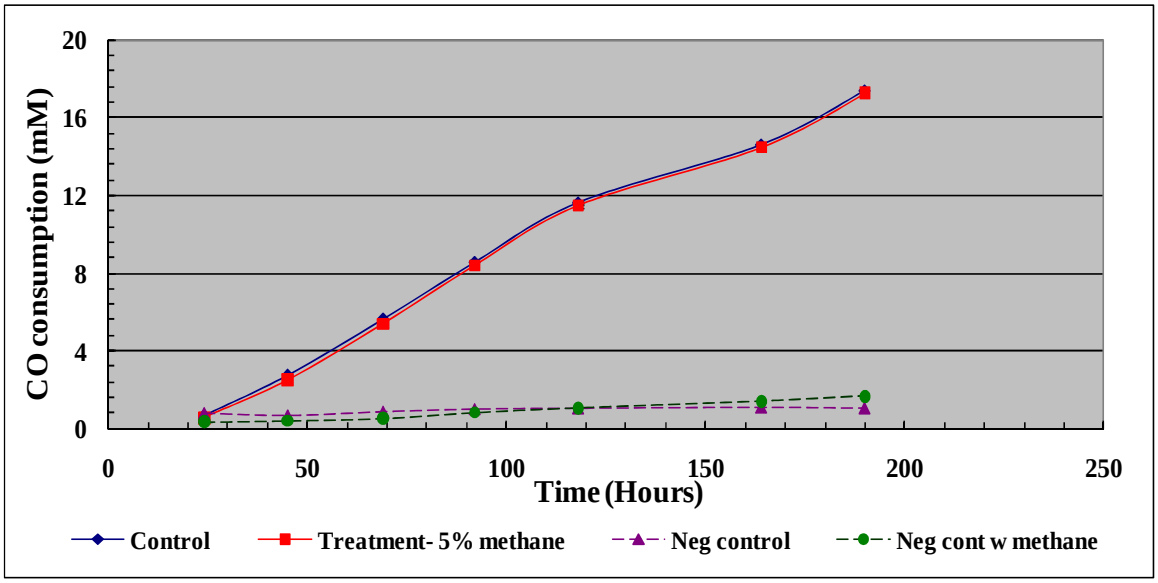

Figure 4.14 CO consumption in different treatments; Each data point is the average of 2 replicates except the negative controls where no replicates was used; Error bars represent standard error.

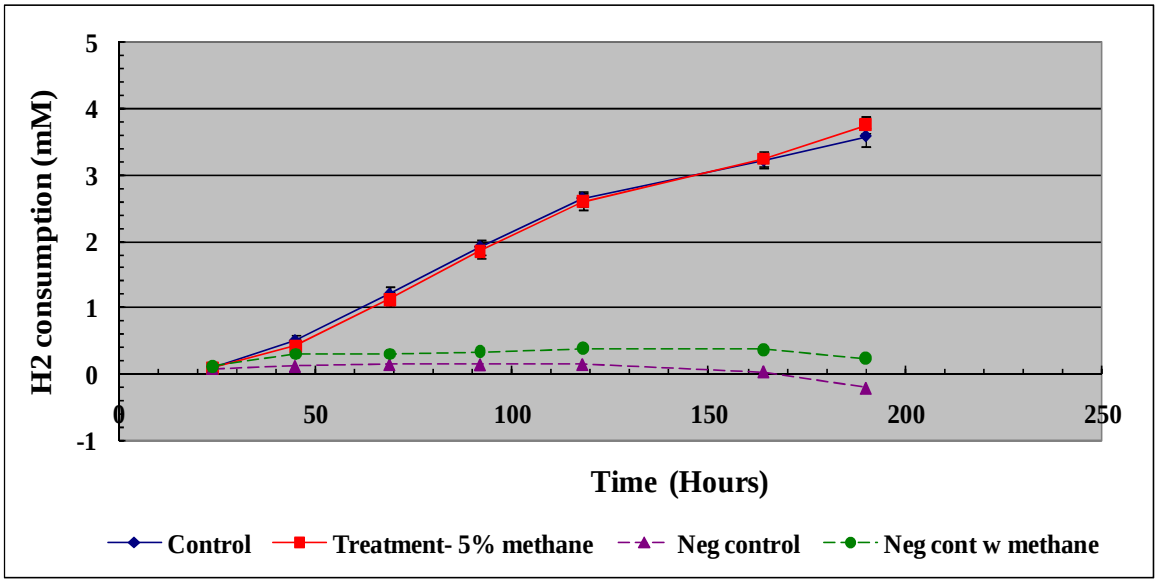

Figure 4.15 H_2 consumption in different treatments; Each data point is the average of 2 replicates except the negative controls where no replicates was used; Error bars represent standard error.

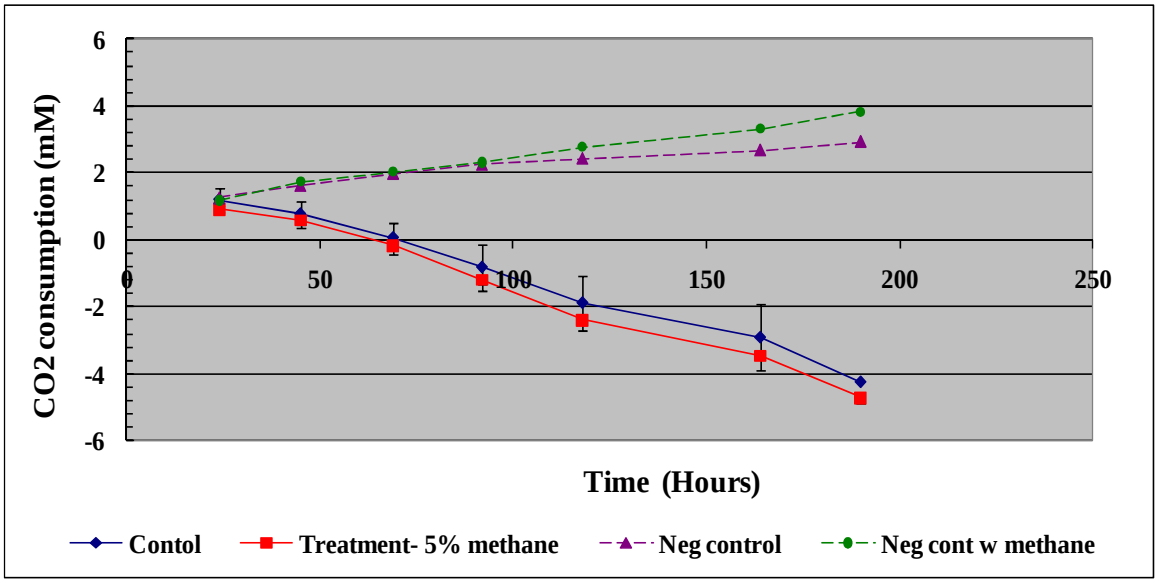

Figure 4.16 CO_2 consumption in different treatments; Each data point is the average of 2 replicates except the negative controls where no replicates was used; Error bars represent standard error. The negative trend shows CO_2 production during P11 fermentations.

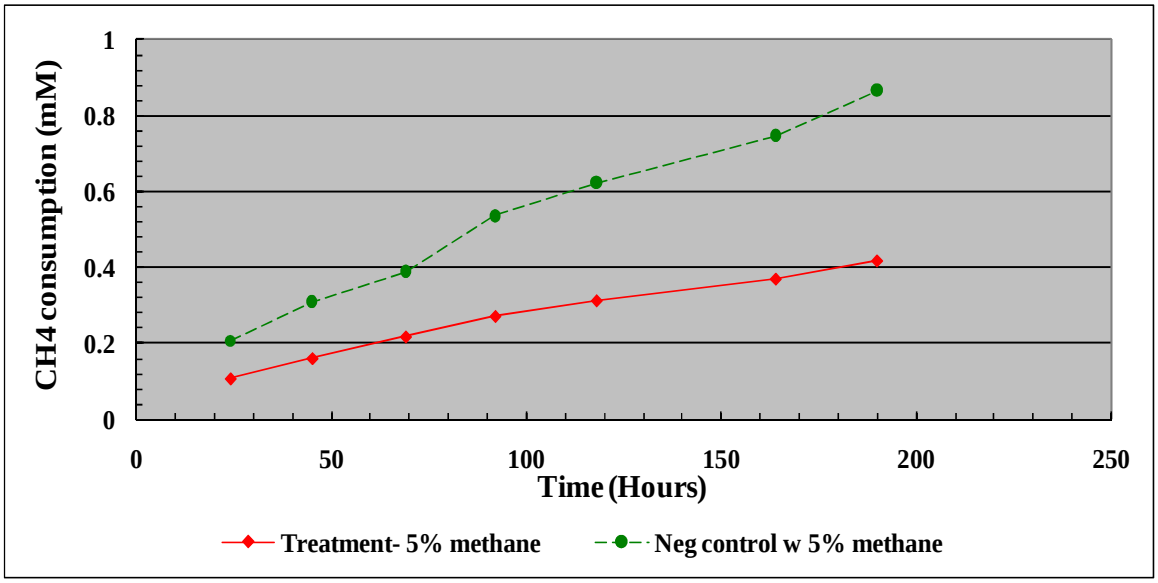

Figure 4.17 CH_4 consumption in different treatments; Each data point is the average of 2 replicates except the negative controls where no replicates was used; Error bars represent standard error.

consumption of these gases were found in both the control and methane treatment, which indicates that methane did not affect the enzymes that are responsible for gas utilization such as carbon monoxide dehydrogenase (CODH) and hydrogenase. CO_2 was produced in P11 fermentations as previously observed, which is evident with a negative trend. An increasing trend of CO_2 and CH_4 apparent consumption for the negative controls is likely due to the solubility of these gases in the media. Furthermore, the small difference in the methane consumption between negative control and the treatment which employed 5% methane indicate that the apparent consumption was due to methane be absorbed by the media not the utilization of methane by the microbes. Similar results in the product formation graphs and gas utilization patterns show that 5% methane did not affect the metabolism of the microbe.

4.3.4 Conclusions

Successful integration of biomass gasification for anaerobic conversion of biomass generated producer gas into ethanol and acetic acid has been demonstrated in lab scale batch reactors. Studies were conducted to investigate the effect of contaminants in switchgrass producer gas and the effect of 5% methane in P11 fermentations. P11 was found to withstand the contaminants of biomass generated producer gas without any loss of cell mass productivity and solvent productivity, which demonstrates the robustness of the microbe. The constituents of producer gas obtained from gasifiers increased ethanol to acetate ratios and produced isopropanol as an additional product. Gas clean up is a critical issue for the successful large scale integration of gasification and fermentation system. The cost of cleaning the producer gas could be reduced if the microbes are found to tolerate and produce more solvents in the presence of contaminants as identified by our

research. Further investigations at molecular level are necessary to assess the reasons behind these metabolic shifts. Besides this, the tolerance limit of each contaminant would help to find the extent of producer gas clean up necessary when different gasifiers and biomass feedstocks are used. Furthermore, this research has also shown that the presence of up to 5% methane in biomass generated producer gas will not affect the fermentation by P11. This is evident from our research as there was no variation in cell growth, product formation and substrate consumption. Thus, the product re-distribution obtained when switchgrass producer gas was employed could not have been due to the presence of methane.

Acknowledgements

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CHAPTER 5

EFFECT OF PHYSICAL PARAMETERS AND EVALUATION OF KINETIC PARAMETERS IN PRODUCER GAS FERMENTATIONS USING *CLOSTRIDIUM* STRAIN P11

5.1 Introduction

Biomass derived feedstocks can provide a renewable and carbon neutral source for ethanol production which has the potential to replace significant amounts of petroleum consumed by the transportation sector of the United States (Khanal 2008; Putsche and Sandor 1996). Primary agricultural crops such as sugarcane and corn are currently the most important feedstocks for bioethanol production and industrial scale production of bioethanol from these feedstocks has been successfully demonstrated by Brazil and the United States (Tsai et al. 2009). These feedstocks are primarily a source of food, which has given rise to many ethical questions like whether corn should be used for food or fuel and the availability of land to grow corn (Anderson et al. 2008). This has led to the development of alternative technologies such as the hydrolysis-fermentation and thermochemical conversion of cellulosic biomass (corn stover, wheat straw, barley straw, rice straw, switchgrass, wood waste, urban waste etc) into ethanol (Ananymous 2009b). One such thermochemical conversion process is gasification. Gasification is a process in which carbonaceous materials (natural gas, naphtha, residual oil petroleum

coke, coal or biomass) react with a gasification medium (air, oxygen and/or steam) at high temperatures (~600-1000 °C) to produce a mixture of gases called synthesis gas (syngas) or producer gas (El-Rub et al. 2004; Huber et al. 2006; Kumar et al. 2009; McKendry 2002b; Spath and Dayton 2003). The producer gas produced by the gasification processes can be converted into numerous products such as heat, electricity, transportation fuels (hydrogen, Fisher-Tropsch diesel, synthetic gasoline) and chemicals using chemical catalysis (El-Rub et al. 2004; Huber et al. 2006). Besides this, microorganisms such as *Peptostreptococcus productus*, *Clostridium ljundahlii*, *Butyribacterium methylotrophicum and Clostridium carboxidivorans* can biologically ferment producer gas into ethanol, acetic acid, acetone and butanol. Biological fermentation of gaseous substrates (or syngas fermentation) have several advantages over chemical catalysis such as higher specificity (Ko et al. 1989), higher yields (Kasteren et al. 2005; Vega et al. 1988b; Worden et al. 1991), lower energy costs (Heiskanen et al. 2007; Kasteren et al. 2005; Vega et al. 1988b; Worden et al. 1991), flexible CO/ H_2/ CO_2 ratios (Huber et al. 2006; Kasteren et al. 2005) and higher resistance to producer gas impurities such as sulfides, tars and other hydrocarbon contaminants (Ahmed 2006; Ahmed and Lewis 2007; Anonymous 1995; Smith et al. 1991; Vega et al. 1990b). However, gasification-fermentation technology is still in its development stage and is less studied than chemical catalysis or hydrolysis-fermentation because of its disadvantages such as slower solvent productivities (Vega et al. 1988b), low solubilities of gaseous substrates in the culture medium (Vega et al. 1988b) and necessity for special design considerations (Barik et al. 1988).

Laboratory scale research in producer gas fermentations are usually performed in serum bottles with producer gas in the headspace that is provided in a fed batch mode. Bottle reactors are generally pressurized, but have a very slow rate of product formation due to low gas solubilities and slow mass transfer. Efforts to increase the rate of product formation has been studied in the past by evaluating environmental conditions like increasing the rate of agitation (Doremus et al. 1985; Henstra et al. 2007) , partial pressure of substrates (Doremus et al. 1985; Ko et al. 1989), headspace (Frankman 2009), pH and heat shock treatments (Gapes et al. 2000; Gapes et al. 1983; Kutzenok and Aschner 1952).

Heat shock is a practice where the cells are exposed to temperatures above their tolerance limit for a brief amount of time to induce sporulation and has been a common practice for culture maintenance for many *Clostridial* strains such as *Clostridium butylicum* (now called *Clostridium beijerinkii*), *Clostridium pasteurianum, Clostridium acetobutylicum* to overcome the strain degeneration phenomena which results in loss of solvent producing abilities by the microbe (Gapes et al. 2000; Gapes et al. 1983; Kutzenok and Aschner 1952; Martin et al. 1983; Spivey 1978). In some studies, heat shock results in sporulation which in turn results in higher solvent production (Jones et al. 1982; Long et al. 1983). Contrary to this, Tracy et al. (2008) found an inverse correlation with sporulation and solventogenesis in C. *acetobutylicum*. Besides sporulation, some reports also suggests the presence of heat shock proteins (hsp74) induced by heat stress are responsible for increased solvent production (Terracciano et al. 1988). Popoutsakis (2005) reported that over expression of heat shock proteins (GroESL) resulted in an increased production and tolerance of butanol in the microbe *C. acetobutylicum*.

Although, *Clostridium* strain P11 (hereafter referred as P11) was known to sporulate occasionally (Huhnke et al. 2006), no research has been performed to observe the effect of heat shock on the bacteria. Preliminary experiments where P11 cells were kept at 5°C above their optimum temperature showed inhibition to growth and product formation during heat treatment but when the heat shock was removed, cells grew normally and attained the same solvent and acid concentrations as the control. Thus, P11 cells were able to withstand the heat treatments and could be sporulating and/or inducing the expression of heat shock proteins during heat treatments. Thus, a hypothesis was made that heat shocks would increase the solvent production. However, the optimum temperature of heat shocking P11 was unknown. Therefore, heat shocking was performed at temperatures of 75°C and 92ºC for 3 min respectively and their performance was compared to the cells which were not treated with heat shocks.

Agitation plays a major role in uniform mixing of the culture medium and optimal supply of oxygen in aerobic fermentations (Aiba et al. 1973). Agitation also plays a very important role in anaerobic fermentations utilizing gaseous substrates because gases such as CO, CO_2 and H_2 are less soluble in water and mass transfer of these substrates to the cells are an important factor in reactor performance (Kapic et al. 2006). In bottle reactors, where no gas sparging is conducted, gas solubility is governed by diffusion and partial pressures. Agitation at higher velocities could increase the mass transfer of gases to the cell surface. Yerushalmi and Volesky (1985) observed that increase in impeller speed in bioreactors from 190 rpm to 340 rpm increased the solvent production rate in *C. acetobutylicum*, but further increase in rpm decreased the solvent production rates. Toma et al. (1991) observed that increased shear could decrease the activity of enzymes in the

TCA cycle in the bacteria *Brevibacterium flavum*. Doremus et al. (1985) studied the effect of agitation and pressure effects in acetone- butanol-ethanol fermentations using *C. acetobutylicum* and observed interesting product distributions. They observed that volumetric productivity of butanol increased with the decrease in agitation rate in a non pressurized system while in pressurized system, agitation had no effect in butanol productivities. Bottle reactors used in our studies were pressurized but CO and H_2 served as carbon and electron sources, unlike ABE fermentations where glucose served as energy source. Thus, there was a necessity to study the effect of agitation in fermentations using gaseous substrates where the mass transfer of CO and H_2 play an important role in reactor performance. Thus, fermentations were carried out at 250 rpm and were compared to fermentations at 150 rpm that served as a control. The hypothesis was that an increase in agitation rates would increase the solvent productivity in pressurized bottle reactors with CO, CO_2 and H_2 as gaseous substrates.

The amount of available gas (otherwise called headspace gas) plays an important role in gaseous substrate fermentations. Although, this physical parameter is less studied, Frankman (2009) carried out experiments with 150 ml and 200 ml headspace gas using P11 and found that higher headspace resulted in faster cell growth (by 2.85 times), higher acetic acid productivity and the onset of ethanol production occurred much earlier (2 days prior to control which had 150 ml headspace). However, no experiments were carried with 200 ml headspace which had showed higher rate of ethanol productivities in preliminary experiments conducted by us (data not shown). Thus, an experiment was conducted with 25 ml culture media (and 257 ml headspace) and was compared with fermentations carried with 100 ml culture media (and 182 ml headspace). Based on the

work performed by Frankman (2009), it was hypothesized that the growth, solvent and acid productivities would be much higher with increased headspace gases.

The present study investigated the effect of physical parameters such as temperature shocks, agitation and headspace on producer gas fermentations with a gas composition of 20% CO, 15% CO_2, 5% H_2 and 60% N_2 in a lab scale using bottle reactors. Kinetic parameters of producer gas fermentation such as product yields, percentage of substrate utilized, percentage of theoretical conversion and gas uptake rates were also calculated for all the studies.

5.2 Materials and methods

5.2.1 Producer gas

Producer gas with a composition of 20% CO, 15% CO_2, 5% H_2 and 60% N_2 was used for all the experiments and served as energy and electron source for the fermentations using P11. Cylindrical gas tanks with the specified composition were purchased from Superior Specialty Gas, Inc (Tulsa, OK). This composition was used because the fluidized bed reactor at OSU produced similar compositions of gas when switchgrass was used as a substrate (Datar 2003).

5.2.2 Microbial catalyst and culture medium

P11, a novel acetogenic bacteria, isolated from a duck pond at the University of Oklahoma was periodically provided by Dr. Ralph Tanner, University of Oklahoma, for the experiments (Huhnke et al. 2006). A defined culture medium was used to cultivate the bacteria that provided essential nutrients for its growth and product formation. One liter of culture media consisted of 30 ml of mineral stock solution (Table 5.1), 10 ml of vitamin stock solution (Table 5.1), 10 ml of trace metal solution (Table 5.1), 1 g of yeast

Components of mineral stock solution	Amount (g/l)	Components of trace metal stock solution	Amount (g/l)	Components of vitamin stock solution	Amount (g/l)
Ammonium chloride	100	Cobalt chloride	0.2	p-(4)-aminobenzoic acid	0.005
Calcium chloride	4	Cupric chloride	0	d-biotin	0.002
Magnesium sulfate	20	Ferrous ammonium sulfate	0.8	Calcium pantothenate	0.005
Potassium chloride	10	Manganese sulfate	1	Folic acid	0.002
Potassium phosphate monobasic	10	Nickel chloride	0.2	MESNA	0.01
		Nitrilotriacetic acid	2	Nicotinic acid	0.005
		Sodium molybdate	0.02	Pyridoxine	0.01
		Sodium selenate	0.1	Riboflavin	0.005
		Sodium tungstate	0.2	Thiamine	0.005
		Zinc sulfate	1	Thioctic acid	0.005
				Vitamin B_{12}	0.005

Table 5.1 Composition of mineral stock solution, trace metal stock solution and vitamin stock solution

extract, 10 g of N-morpholinoethanesulfonic acid (MES) buffer, 10 ml of 4% cysteine sulfide solution and 1 ml of 0.1% resazurin solution. Mineral solution provides the source of mineral ions that are crucial as cofactors of various enzymes, membrane transport and as components of molecules and structural complexes (Cote and Gherna 1994). Trace metals play an important role in anaerobiosis as most of the metals acts as cofactors to metalloenzymes (Cote and Gherna 1994; Tanner 1997). Similarly, vitamins also play an important role in catalytic functions as coenzymes, prosthetic groups (Cote and Gherna 1994). MES is a buffering agent which is used to avoid excessive pH fluctuation during the fermentation process as external pH controllers were not used. Low redox potentials are necessary for anaerobic processes (Wiegel et al. 2006) was achieved by the addition of cysteine sulfide solution. Besides this, cysteine sulfide solution also scavenges any dissolved oxygen if present in the media. Resazurin solution acted as a redox indicator. All the chemicals were purchased from Sigma Chemical Company, St. Louis, Missouri except yeast extract that was purchased from Difco laboratories, Detroit, Michigan.

5.2.3 Preparation of culture medium and batch studies

All the batch fermentation studies were performed in 250 ml serum bottles with 100 ml of culture media (except where another media volume is mentioned). The culture media was prepared by mixing all its components (mineral stock solution, trace metal solution, vitamin solution, MES, yeast extract and resazurin) in the appropriate amount of deionized water in a round bottomed flask. The pH of the culture medium was then adjusted to 6.1 using 2 N potassium hydroxide solution. The culture media was then made anaerobic by boiling it in a microwave and then briskly passing nitrogen through the media. The flask was sealed and taken into an anaerobic glove box (Coy Laboratory

Products Inc., Grasslake, MI) where appropriate volume of media was dispensed into serum bottles which were then sealed prior to removal from the glove box. The bottles were then sterilized in an autoclave (Primus Sterilizer Co. Inc, Omaha, NE) at 121 ºC for 20 min. After autoclaving, the serum bottles were then brought to room temperature and 1 ml of 4% sterile cysteine sulfide solution per 100 ml of media. The bottles were then purged with producer gas up to a pressure of 239.25 KPa (or 34.7 psia) and inoculated with a 10% inoculum (unless otherwise mentioned) from actively growing cultures. The actively growing cells are obtained by sub-culturing twice. Sub-culturing was carried out in duplicates. Each stage of sub-culturing is referred as a passage. Inoculum transfer from one passage to the other was performed when the cells reached mid exponential phase. The bottle reactor in which the microbes grew faster was chosen for inoculum transfers into next passage as it was expected to have more healthy cells than the bottle reactor that grew slower. All inoculum transfers were done from the same reactors, thereby, minimizing the culture variation from reactor to reactor and from one treatment to another. The media compositions and the method of culture media preparation remained the same as the experimental procedure during the subculture stages. After inoculation the batch reactors were placed in a warm room where the temperature was maintained at 37ºC on an orbital shaker (Innova 2100, New Brunswick Scientific, Edison, NJ) at 150 rpm. Cell concentration, pH, ethanol concentration, acetic acid concentration and producer gas concentrations were analyzed at regular intervals. The overall sketch of the three experiments is shown in the next section. Throughout the experiments, producer gas was fed at every 24 hour intervals.

5.2.3 Analytical methods

All the analytical methods are the same as described in Chapter 4.

5.3 Results and Discussion

5.3.1 Batch studies – Effect of heat shock treatments

The experimental outline of the heat shock experiment is shown in Fig. 5.1. The cells were sub-cultured in one stage (or in the first passage) to get actively growing cells. During the second passage, P11 culture was subjected to heat shocks at two different temperatures: 92°C for 3 min and 75ºC for 3 min and was compared to the cells that were not subjected to heat treatment. Heat shocks were provided by placing the bottle reactors (that had 25 ml of culture media with freshly inoculated culture) in an agitated hot water bath for uniform heat transfer throughout the culture media. The batch reactors were cooled down immediately by placing in a water bath maintained at room temperature and were pressurized to 239.25 KPa (or 34.7 psia). The reactors were then incubated at 37°C at 150 rpm. After heat shock, when the cells reached mid exponential phase in passage 2, they were transferred to the next passage where 100 ml of culture media was used to grow them. It was hypothesized that both the heat shock treatments would have a longer lag phase because heat shocking would kill the vegetative cells. It was also hypothesized that the cells that were heat shocked will produce more ethanol compared to the control. 5% inoculum transfers were carried out in all passages.

Cell growth and pH: The growth and pH profiles of different treatments are shown in Figs. 5.2 and 5.3. The treatments that were subjected to heat shock had a lag phase of 20 h in the second passage (data not shown). Higher temperatures had decreased the amount of vegetative cells and many spores were observed when a 20 h sample was

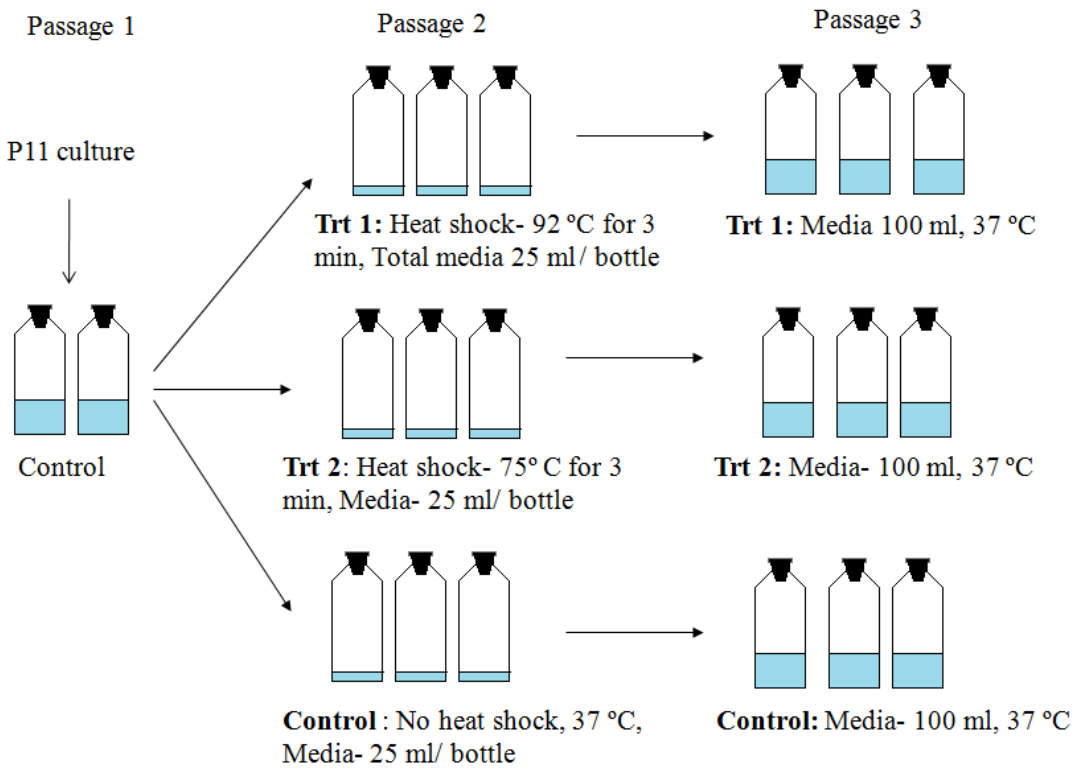

Figure 5.1 Experimental outline for heat shock experiment

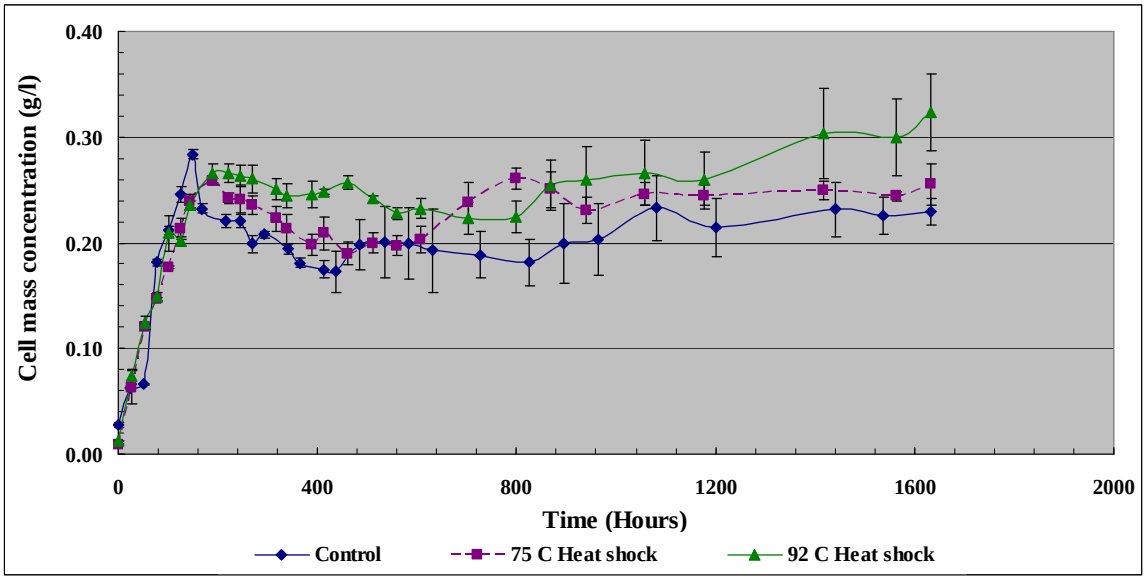

Figure 5.2 Cell growth in different treatments. Each data point is the average of 3 replicates; Error bars represent standard error.

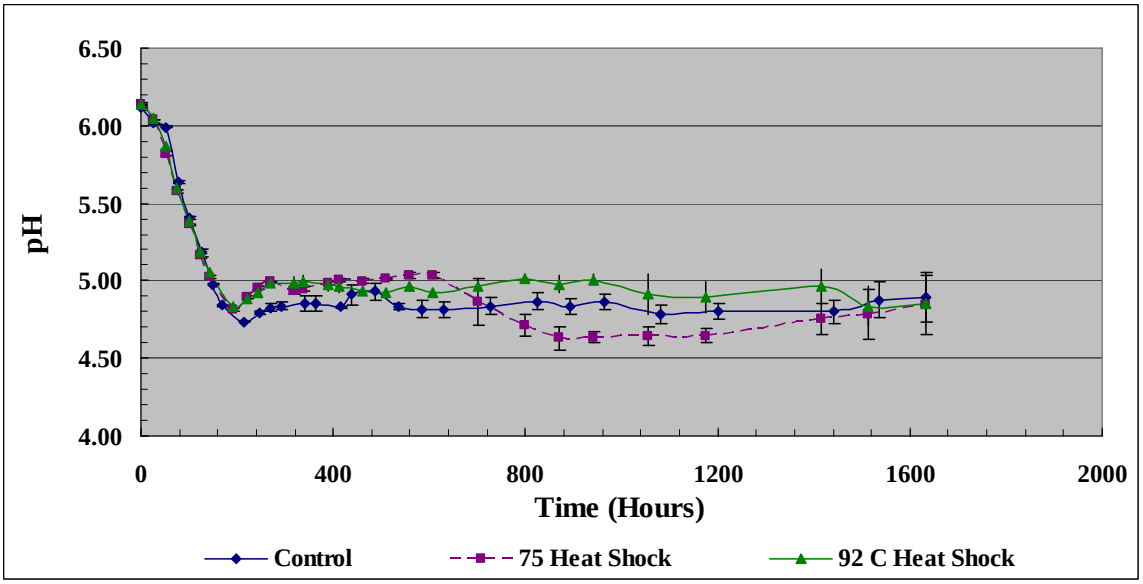

Figure 5.3 pH profile in different treatments. Each data point is the average of 3 replicates; Error bars represent standard error.

taken and spore stained using malachite green using the method described by Gerhardt et al. (1981). In the third passage, growth of P11 occurred rapidly in the first 200 h followed by a slight decrease in cell densities. This observation was similar to other fermentations using P11. There was a secondary cell growth observed in all the treatments after 600 h (approximately). The secondary growth phase was also associated with production of acetic acid and uptake of CO and H_2. Multi-growth phases similar to our observations were also observed in *C. acetobutylicum* (Tracy et al. 2008). The reason for secondary growth is unknown but it could be possible that the cells need more energy for cellular metabolism and acetic acid production during this stage produces ATP. The final cell mass concentrations were the highest for the cells that were treated at 92ºC followed by 75°C and control cells (where the cells were not subjected to any heat treatment). Media pH dropped rapidly from 6.1 to 4.7 due to acetic acid production (Fig. 5.5). The break point in the pH profile (an indication of shift in the metabolism from acidogenesis to solventogenesis) was found to be 25 h earlier with both the heat treatments (190 h) than compared to the control (215 h). However, the final pH in the different treatments was almost the same in all the treatments.

Product profiles: Acetic acid (Fig. 5.5) is observed to be growth related as it is involved with ATP production which is necessary for cellular replication and metabolism (Henstra et al. 2007; Imkamp and Muller 2007). Ethanol (Fig. 5.4) is a secondary metabolite in P11 fermentations. Hence, ethanol production starts only after 200 h (approximately). But, from Fig. 5.4 it can be observed that the rate of ethanol production was higher with the heat treatments (both 75ºC and 92ºC) than the control. For example,

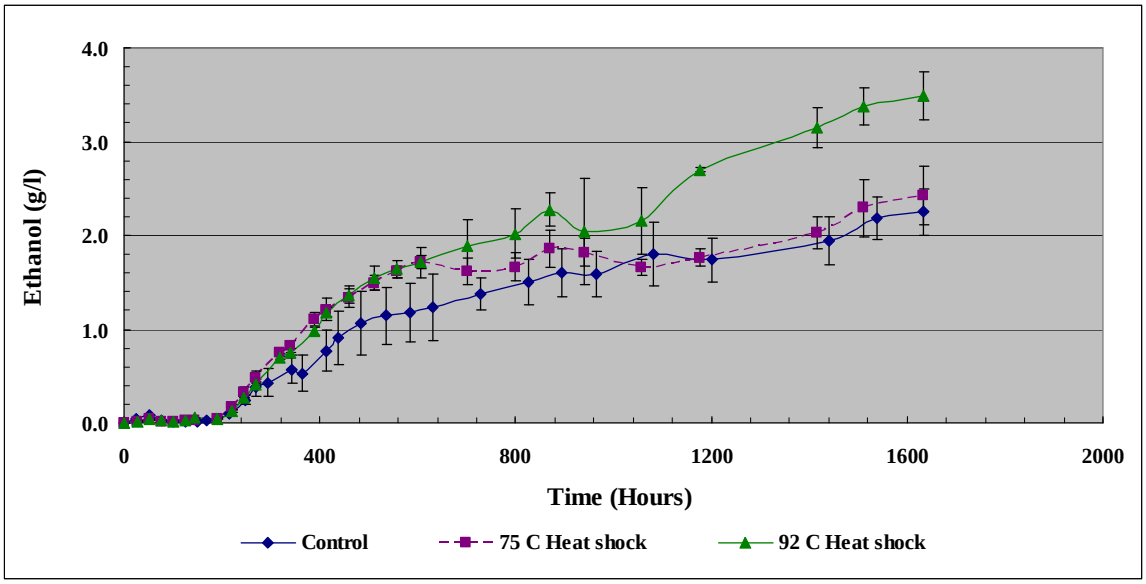

Figure 5.4 Ethanol production in different treatments. Each data point is the average of 3 replicates; Error bars represent standard error.

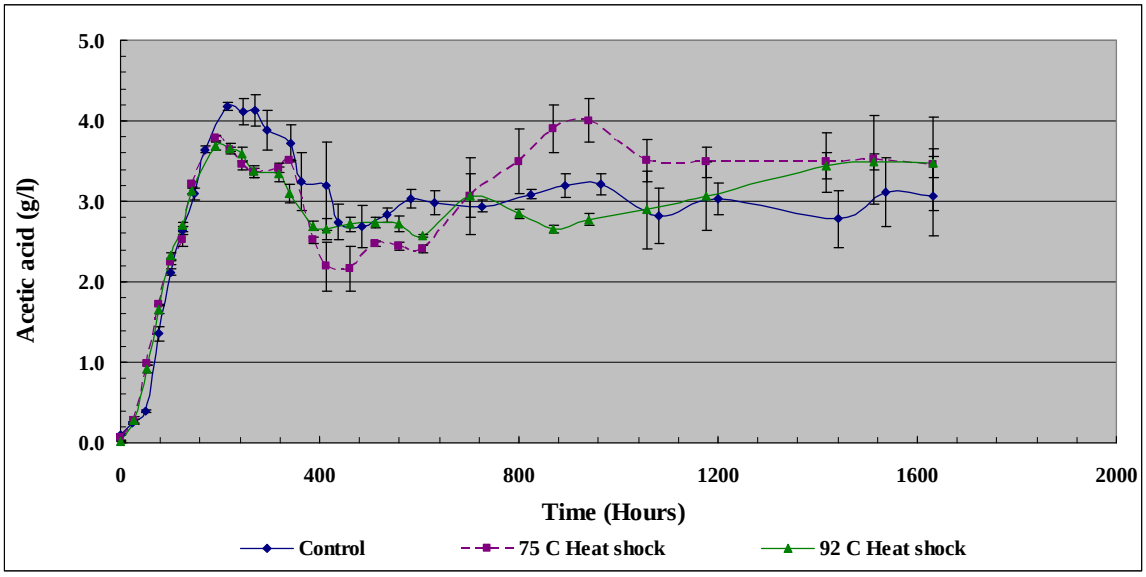

Figure 5.5 Acetic acid production in different treatments. Each data point is the average of 3 replicates; Error bars represent standard error.

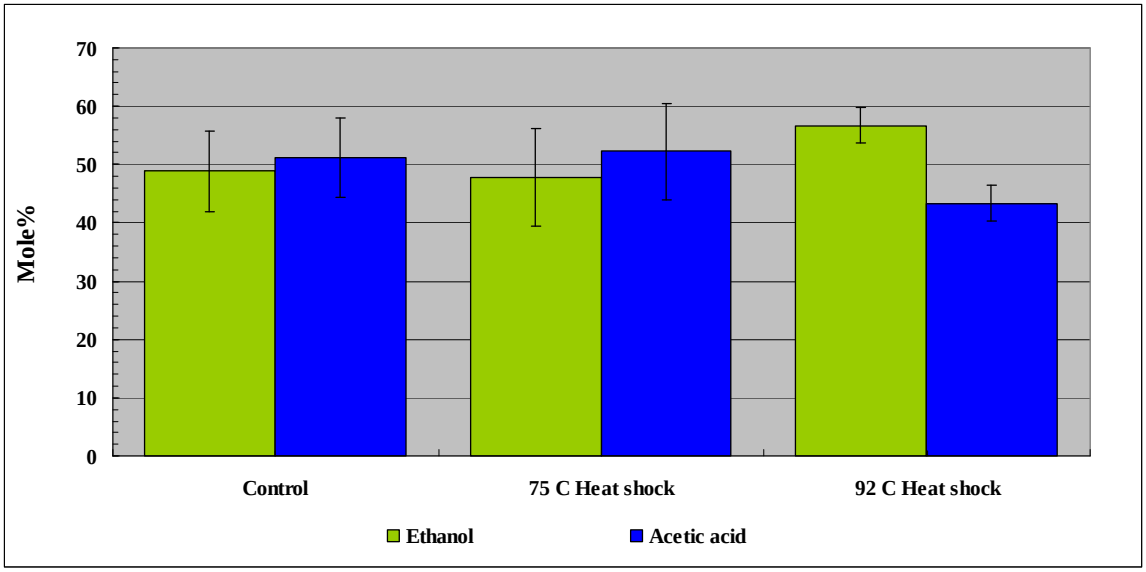

Figure 5.6 Molar percentages of products formed in different treatments. Each data point is the average of 3 replicates; Error bars represent standard error.

at the end of 600 h, ethanol concentrations in both the heat treatments was 1.7 g/l, whereas, the control had only 1.2 g/l of ethanol. After 600 h, the cells that were treated at 75ºC entered a secondary growth phase and produced acetic acid and hence the rate of ethanol production decreased, thus, the control and 75°C heat shock treatment had similar concentrations of ethanol at the end of fermentation. Cells exposed to 92ºC produced 55% more ethanol than the control (3.5 g/l in the 92ºC heat shock treatment compared to 2.3 g/l in control and 2.4 g/l in 75ºC shocks) at the end of fermentation. In the same way, the cells that were exposed to 92°C and 75°C shock produced the highest acetic acid concentrations (3.5 g/l) compared to control that produced only 3.1 g/l of acetic acid. Higher ethanol and acetic acid production observed with the 92°C heat treatment could be due to an up-regulation of enzymatic activities which has to be tested.

The acetic acid concentrations decreased after the first 200 h (growth phase) in all the treatments, meaning the acetic acid could have been possibly converted into ethanol. The molar distribution of products (Fig. 5.6) remained the same in the control and 75°C heat shock (with approximately 48% ethanol and 52% acetic acid) but the 92ºC heat shock treatment had 57% ethanol and 43% acetic acid. The ethanol to acetate ratio (molar basis) for 92°C heat shock was 1.0 compared to 0.7 with control and 75ºC heat shock. Higher ethanol concentrations in the 92ºC heat shock treatment could be a result of sporulation (Jones et al. 1982; Long et al. 1983) and/or the increased expression of heat shock proteins (Papoutsakis 2005; Terracciano et al. 1988).

Gas consumption and kinetic parameters: The consumption of gaseous substrates CO, H_2 and CO_2 are shown in Figs. 5.7, 5.8 and 5.9, respectively. Rapid consumption of CO and H_2 and production of CO_2 were observed during growth phase, which could be a

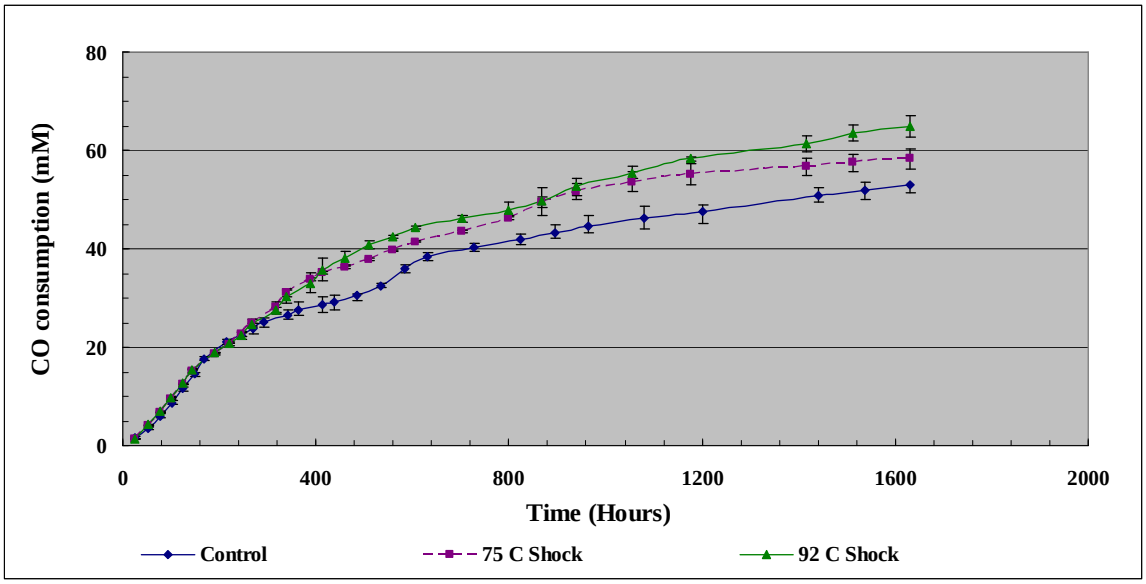

Figure 5.7 CO consumption profile in different treatments. Each data point is the average of 2 replicates; Error bars represent standard error.

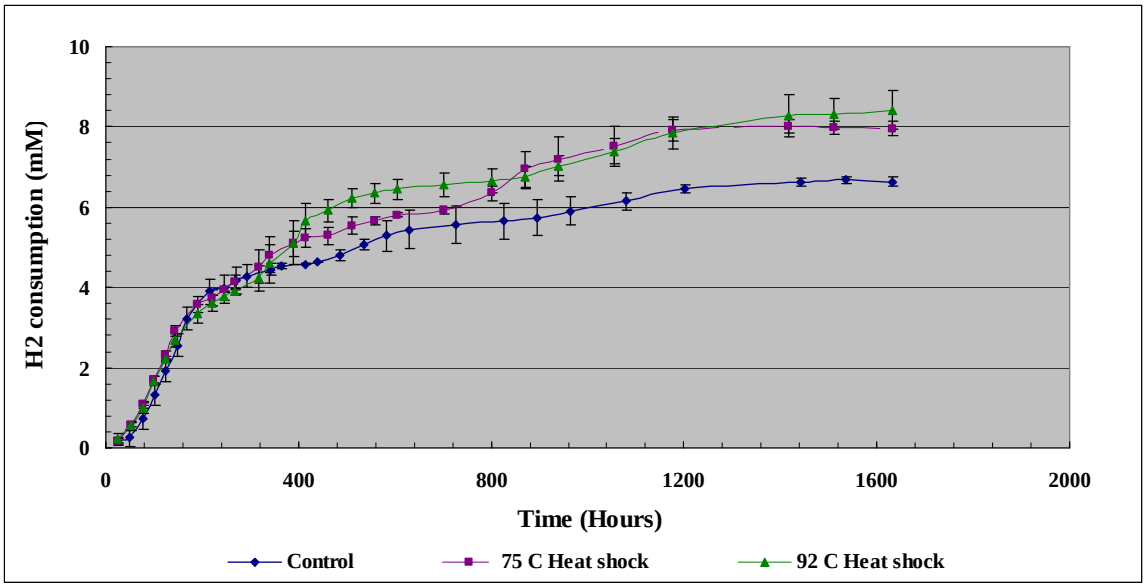

Figure 5.8 H_2 consumption profile in different treatments. Each data point is the average of 2 replicates; Error bars represent standard error.

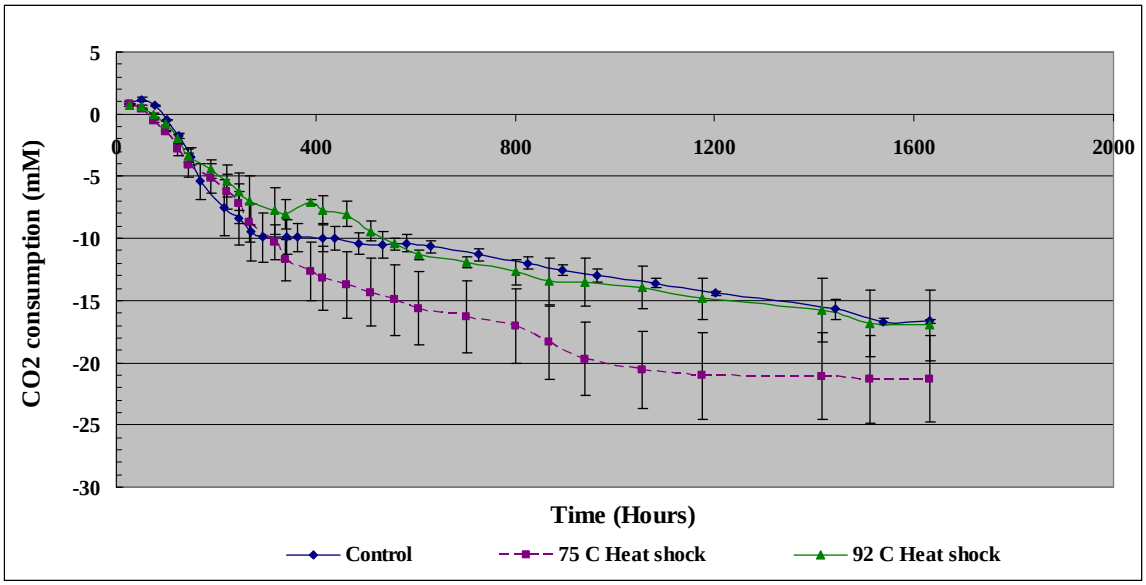

Figure 5.9 CO_2 consumption profile in different treatments. Each data point is the average of 2 replicates; Error bars represent standard error. The negative trend shows CO_2 production.

result of reactions 5.1, 5.2 and 5.3. CO can act as a carbon and electron source while H_2 serves as an electron source only. Thus, P11 shows preference to CO when a mixture of CO and CO_2 is provided like other acetogens such as *P. productus* (Vega et al. 1988a) and *C. carboxidivorans* (Shenkman 2003). The consumption of CO and H_2 slowed down during the stationary phase when ethanol was being produced. In all the treatments, decrease in acetic acid was observed, which could be a result of reduction of acetic acid into ethanol as shown in reaction 5.5.

$$2CO + 4H_2 \rightarrow C_2H_5OH + H_2O \quad (5.1)$$

$$CO + H_2O \rightarrow CO_2 + H_2 \quad (5.2)$$

$$3CO + 3H_2 \rightarrow C_2H_5OH + CO_2 \quad (5.3)$$

$$2CO + 2H_2 \rightarrow CH_3COOH \quad (5.4)$$

$$CH_3COOH + 2\ H_2 \rightarrow C_2H_5OH + H_2O \quad (5.5)$$

$$5CO + 7H_2 \rightarrow 2C_2H_5OH + CO_2 + H_2O \quad \text{(Overall reaction)} \quad (5.6)$$

CO and H_2 consumption (in moles) was the highest in the 92°C heat shock, treatment followed by 75ºC heat shock and control. This also resulted in higher CO and H_2 utilization and higher molar yields of ethanol ($Y_{ETOH/CO}$) with the 92°C heat treatment (Table 5.2). From reaction 5.3, 1 mole of ethanol is produced from 3 moles of carbon in CO, thus making the theoretical maximum of ethanol from CO as 0.333 moles of ethanol/ mole of CO. Based on these calculations, the % theoretical conversion of ethanol from CO (Actual $Y_{ETOH/CO}$ / Theoretical $Y_{ETOH/CO}$) were calculated and found to be higher for the treatment with 92ºC heat shock treatment (Table 5.2). Also, the specific ethanol formation rate expressed in millimoles of ethanol produced per hour per gram of cells was found to be higher with 92ºC heat shock treatment (Table 5.2).

Kinetic parameter	Control	75 °C shock	92 °C shock
% CO utilized	45.96%	52.55%	58.49%
% H_2 utilized	22.98%	28.72%	30.36%
Yield (ETOH/CO) $Y_{ETOH/CO}$ (moles of ethanol/mole of CO)	0.062	0.063	0.079
Yield (AA/CO) $Y_{AA/CO}$ (moles of acetic acid/mole of CO)	0.087	0.085	0.076
% Theoretical conversion (Actual $Y_{ETOH/CO}$ / Theoretical $Y_{ETOH/CO}$)	18.57%	20.30%	25.15%
% Theoretical conversion (Actual $Y_{AA/CO}$ / Theoretical $Y_{AA/CO}$)	17.34%	17.09%	15.24%
Specific CO uptake rate (mM CO/ hr/ g cells)	2.61	2.58	2.27
Specific H_2 uptake rate (mM H_2/ hr/ g cells)	0.33	0.35	0.29
Specific CO uptake rate (mM CO/ hr/ g cells) (First 510 hours)	4.39	5.29	4.71
Specific H_2 uptake rate (mM H_2/ hr/ g cells) (First 510 hours)	0.68	0.77	0.72
Specific ethanol formation rate (mM ethanol/ hr/ g cells)	0.183	0.179	0.197

Table 5.2 Kinetic parameters of P11 fermentation during heat shock experiment.

Ethanol production continued even after H_2 consumption ceased after 1400 h (approximately) in all the treatments. Possibly, CO could be acting as electron source for the reduction reactions involved in ethanol formation (Ahmed and Lewis 2007). CO_2 production was almost the same in 92ºC heat shock treatment and control but higher in 75ºC heat shock treatment. This could be due to the abrupt secondary growth that was observed in 75ºC heat shock treatment after 600 h (approximately).

Final concentrations of acetic acid in the fermentation media were similar in all the experimental conditions, but the yield of acetic acid ($Y_{AA/CO}$) was lower in the 92 °C heat shock treatment as the CO consumed were higher (Table 5.2). Also, since the final cell mass concentration was higher in the 92°C heat than the 75°C shock treatment and the control that resulted in lower overall CO and H_2 utilization rates (Table 5.2). But, when closely seen in the CO and H_2 consumption curves, most of the gas consumption occurred in the first 500 h of fermentation. Thus, when the gas utilization rates were calculated for the first 500 h, the 92ºC heat shock treatment higher CO and H_2 utilization rates than the control (Table 5.2).

To summarize, both the heat shock treatments were found to increase the percentage of gas utilization of CO and H_2, but the 92°C heat shock treatment produced the highest ethanol. Gapes et al. (2000) showed 95ºC heat shocks for 2.5 min gave the highest butanol to acetone yield with *C. beijerinkii* B592. Although, the morphological effect during the fermentation were not observed by Gapes et al. (2000), Jones at al. (1982) had found that the sporulation was related to solventogenesis. Later, Rogers et al. (2006) showed that solventogenesis and sporulation were activated by a common regulatory elements in the microbes *C. acetobutylicum* and *C. beijerinckii*. Besides

sporulation, some research also suggests the induction of heat shock proteins (hsp74) during heat stress that were responsible for solvent production (Terracciano et al. 1988). Popoutsakis (2005) reported that over expression of heat shock proteins (GroESL) resulted in an increase in production and tolerance of butanol in the microbe *C. acetobutylicum*. Although, our study did not look into the morphological alterations of P11 during fermentation, we did confirm that a 20 h sample after heat shocking had spores. Further investigation of the cause of metabolic shifts in the 92ºC heat shock treatment is necessary. This research has shown that the solvent yields and gas uptake rates could be increased by heat shocking P11 cells during the subculture stages at 92°C for 3 min.

5.3.2 Batch studies - Effect of increased agitation speed

The overall experimental outline is shown in Fig. 5.10. P11 cells were sub-cultured in duplicate before transferring to the third passage where the fermentation kinetics is compared between two different agitation speeds: 250 rpm and 150 rpm (control). Both the treatments were done in duplicate. As agitation is one of the important factors in the mass transfer of gaseous substrates in the culture media (Kapic et al. 2006; Riet and Tramper 1991; Yerushalmi and Volesky 1985), it was hypothesized that producer gas fermentation at 250 rpm would increase the solvent productivity in comparison to the fermentation at 150 rpm (control).

Growth and pH: The growth and pH profiles of different treatments are shown in Figs. 5.11 and 5.12, respectively. A small lag phase of 19 h was initially observed, which was followed by rapid cell growth in both the treatments. P11 culture at 250 rpm reached maximum cell concentration (0.47 g/l) 73 h earlier than the cells that were at 150 rpm.

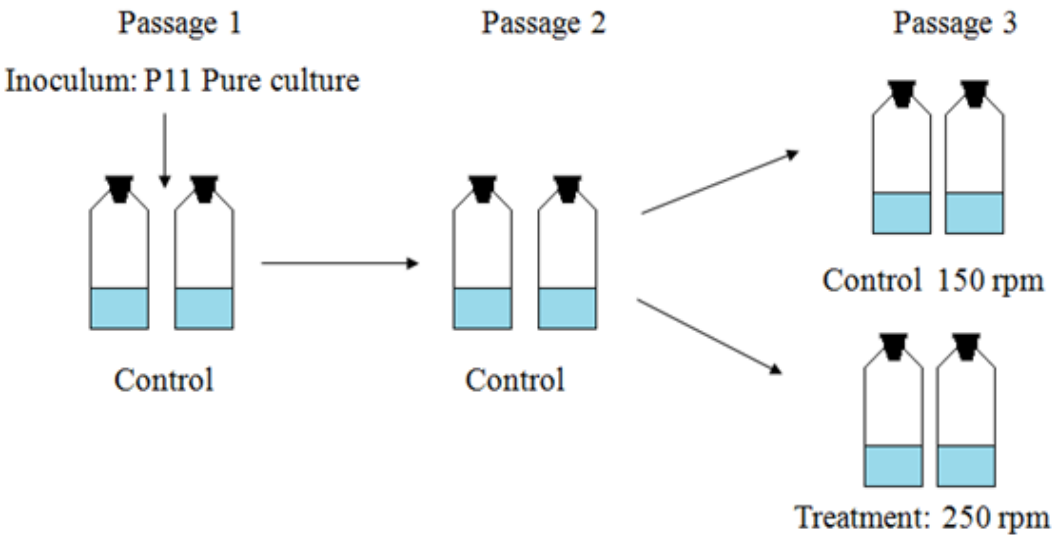

Figure 5.10 Experimental outline of agitation speed experiment.

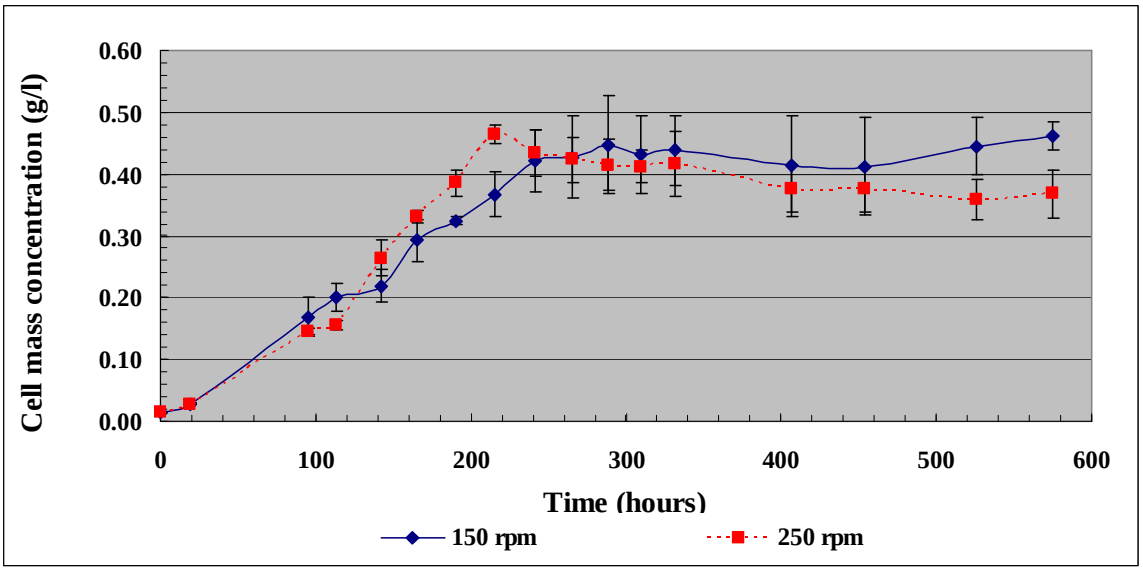

Figure 5.11 Cell growth in different treatments. Each data point is the average of 2 replicates; Error bars represent standard error.

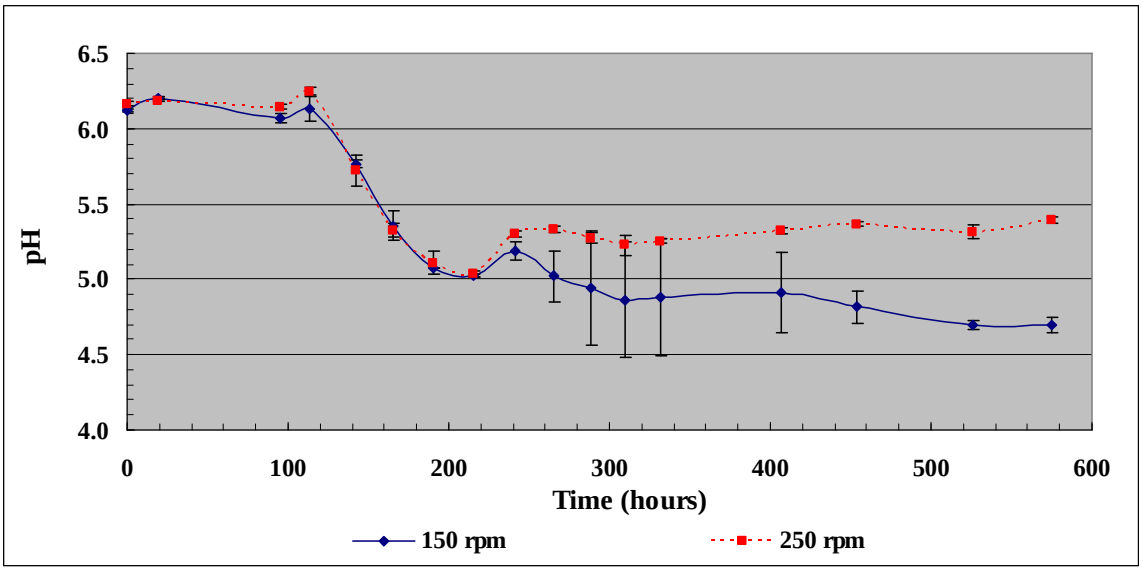

Figure 5.12 pH in different treatments. Each data point is the average of 2 replicates; Error bars represent standard error.

There was a decline in cell mass after P11 culture reached maximum cell mass concentration at 250 rpm, but the control (cells at 150 rpm) was constant after the maximum was reached. Media pH dropped rapidly from 6.1 to 5 in both the treatments, with the control, a further decrease of pH from 5 to 4.70 occurred.

Product profiles: Acetic acid and ethanol were the products formed during the producer gas fermentation using P11. Acetic acid (Fig. 5.14) was found to be a growth related product and ethanol (Fig. 5.13) to be a non-growth associated product. Both products' (ethanol and acetic acid) concentrations were higher with cells that were agitated at 150 rpm than 250 rpm. Ethanol production initiated 25 h earlier with the cells agitated at 250 rpm compared to control, but ethanol production stopped abruptly after 240 h (approximately). The cause of the early initiation of ethanol could be due to the early cessation of acetic acid formation (at 165 h) when the cells were agitated at 250 pm while the control reached maximum acetic acid concentration at 240 h. Ethanol concentration decreased by 50% when P11 cells were agitated at 250 rpm as compared to 150 rpm (1.8 g/l at 150 rpm and 0.9 g/l at 250 rpm of ethanol were obtained). Similarly, the maximum acetic acid concentrations were 52% lower when cells were agitated at 250 rpm (4.2 g/l at 150 rpm and 2 g/l at 250 rpm). In the 150 rpm treatment, the acetic acid concentration gradually decreased from 4.2 g/l to 2.7 g/l towards the end of fermentation. This could have resulted in ethanol production by the reduction of acetic acid. Similar observations of decreasing acetic acid concentrations were observed with other fermentations (Chapter 4). Lower concentrations of acetic acid and ethanol at 250 rpm indicate that higher agitations could be detrimental to the product formation. The cessation in ethanol formation after 250 h with complete cessation of CO and H_2 uptake

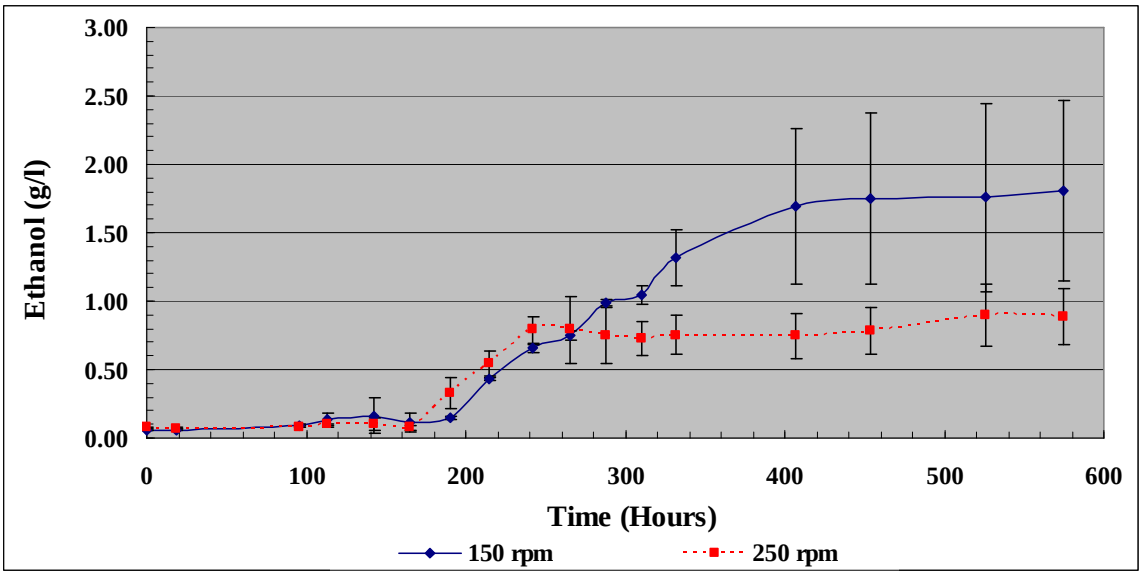

Figure 5.13 Ethanol concentrations in different treatments. Each data point is the average of 2 replicates; Error bars represent standard error.

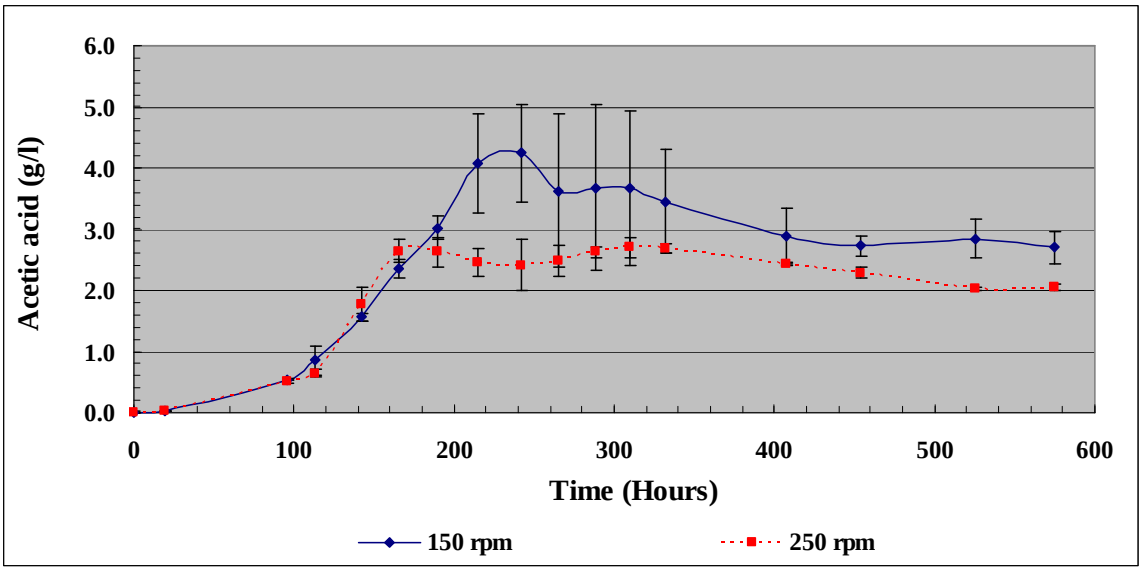

Figure 5.14 Acetic acid concentrations in different treatments. Each data point is the average of 2 replicates; Error bars represent standard error.

(results described in next section) could be possibly due to the shear sensitivity of P11 cells to higher agitation rates. Toma et al. (1991) observed that the activity of enzymes involved in the TCA cycle decreased due to the increase in agitation rates in the microbe *B. flavum*. Enzymes such as CODH and hydrogenase involved in the acetyl-CoA pathway could be affected due to the shear.

Gas consumption and kinetic parameters: CO and H_2 are the major sources of energy and electrons in producer gas fermentation using P11. Rapid consumption of CO and H_2 (Figs. 5.15 and 5.16) occurred during the growth phase of P11 in both the treatments. Besides ethanol and acetic acid, CO_2 (Fig. 5.17) was a product of producer gas fermentations. Lower ethanol and acetic acid concentrations were due to the inhibition in CO and H_2 uptake and CO_2 production after 250 h (approximately). No signs of metabolism was shown by the cells agitated at 250 rpm (after 250 h) leading to a conclusion that P11 cells could be shear sensitivity of to higher agitation rates. This resulted in lower percentage utilization of CO and H_2, lower CO and H_2 uptake rates, lower specific ethanol formation rate and lower molar yields of ethanol ($Y_{ETOH/CO}$) when P11 cells were agitated at 250 rpm (Table 5.3). The kinetic parameters obtained at 150 rpm (control) were similar to the previous experiment. The molar ratio of ethanol to acetic acid at 150 rpm was 0.8 while at 250 rpm was 0.6 clearly indicating the failure to produce reduced end products such as ethanol.

Our hypothesis that higher agitation rates would increase the product concentrations was disproved. P11 cells obtained similar cell mass concentrations in both conditions in the first 200 h, but after 250 h the CO and H_2 consumption and ethanol production completely stopped indicating the possibility of cells shear sensitivity. Our

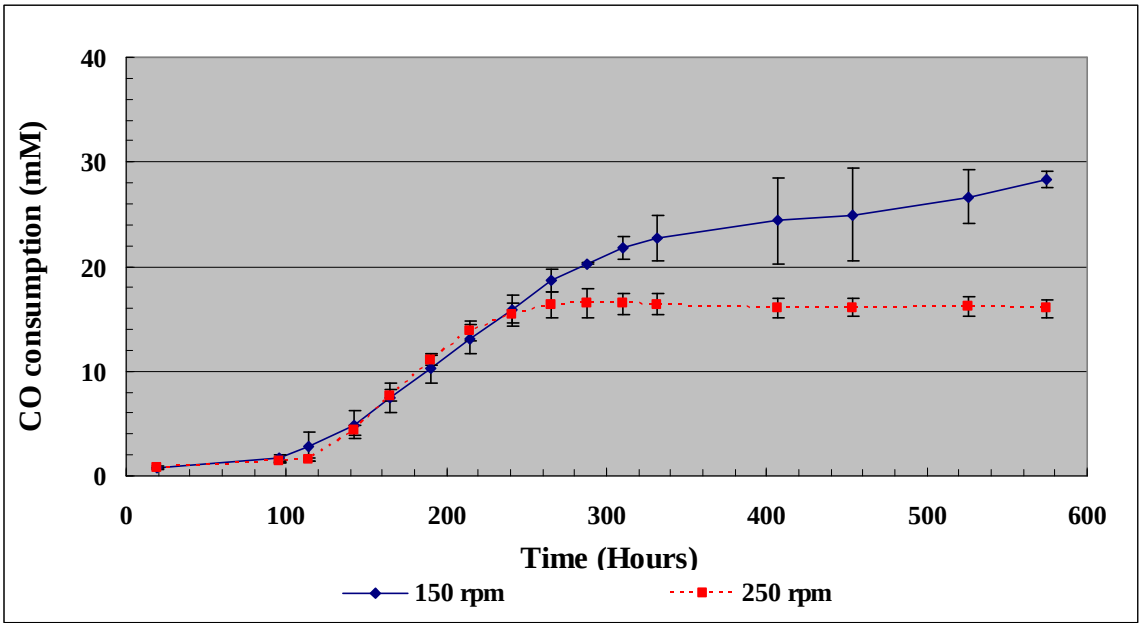

Figure 5.15 CO consumption profile in different treatments. Each data point is the average of 2 replicates; Error bars represent standard error.

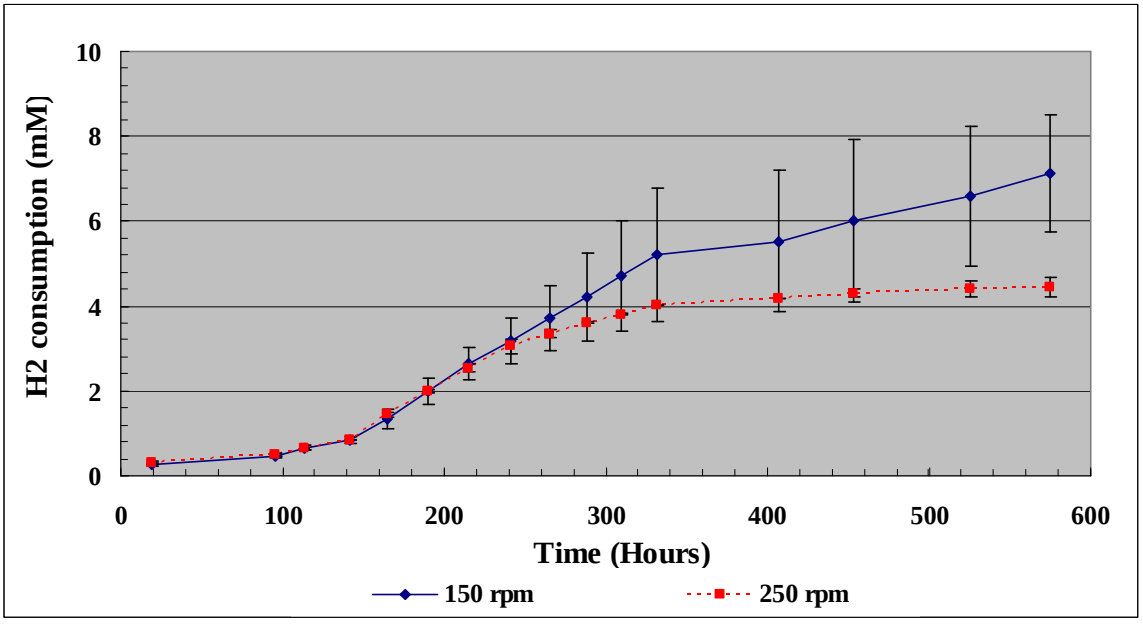

Figure 5.16 H_2 consumption profile in different treatments. Each data point is the average of 2 replicates; Error bars represent standard error.

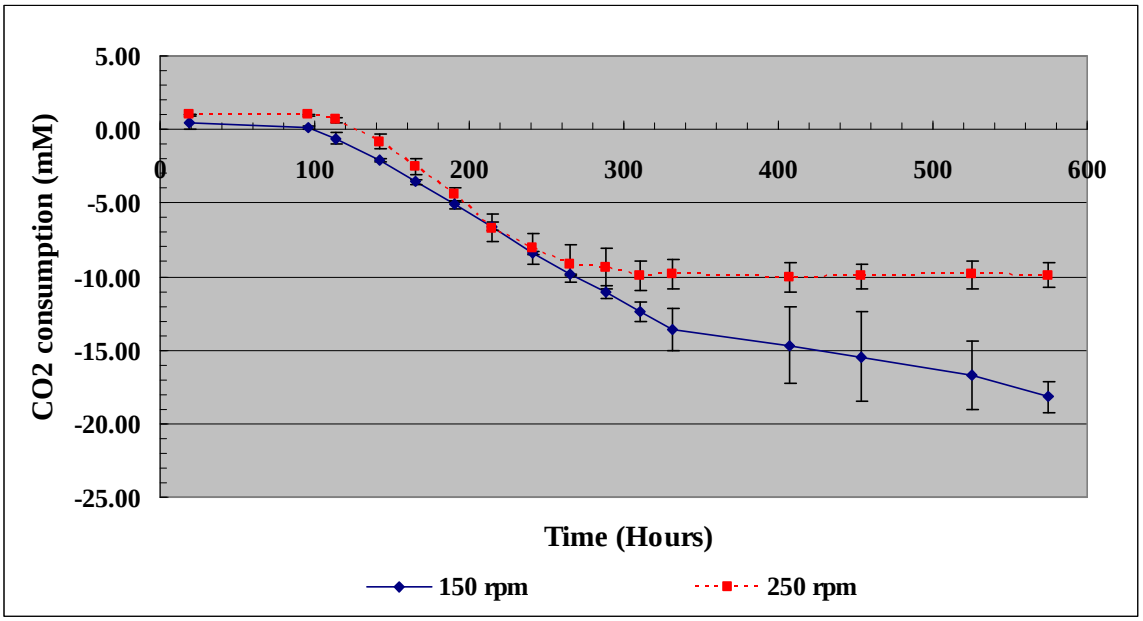

Figure 5.17 CO_2 consumption in different treatments. Each data point is the average of 2 replicates; Error bars represent standard error. The negative trend shows CO_2 production.

observations are similar to Yerushalmi and Volesky (1985) that noted a decrease in the formation of solvents and gases without affecting the cell mass concentrations of *C. acetobutylicum* when the rpm was increased above 340 rpm. Further research has to be performed at agitation rates between 150 rpm and 250 rpm in order to confirm if there is any negative correlation between agitation rates and product profiles. Our research supports the results reported by Doremus et al. (1985) that agitation had no effect in butanol productivities in a pressurized system with *C. acetobutylicum*. The agitation effect on non-pressurized systems like bioreactors is believed to behave differently than bottle studies and will need further investigation with agitation rates. Although, it is believed that there could be advantages with increasing agitation rates in bioreactors up to a particular limit, as reported by Yerushalmi and Volesky (1985), important considerations like shear sensitivity will have to be taken into account during optimization of agitation rates.

5.3.3 Batch studies - Effect of increased headspace

The experimental outline of the headspace optimization experiment is shown in Fig. 5.18. The headspace was increased by decreasing the volume of culture media from 100 ml in the control to 25 ml in the treatment with increased headspace. The cells were sub-cultured in two passages with an increased headspace before inoculating the cells into the experimental stage. 5% inoculum transfers were made in all passages. In the third passage, the performance of increased headspace (256.5 ml) was compared to normal headspace (178 ml) with respect to product concentrations, product yields, solvent productivities, producer gas uptake rates and ethanol formation rates. Due to lesser culture volume with increased headspace, liquid samples were withdrawn once every 72

Kinetic parameter	150 rpm	250 rpm
% CO utilized	47.40%	26.78%
% H_2 utilized	43.74%	26.25%
Yield (ETOH/CO) $Y_{ETOH/CO}$ (moles of ethanol/mole of CO)	0.123	0.116
Yield (AA/CO) $Y_{AA/CO}$ (moles of acetic acid/mole of CO)	0.174	0.227
% Theoretical conversion (Actual $Y_{ETOH/CO}$ / Theoretical $Y_{ETOH/CO}$)	36.94%	34.76%
% Theoretical conversion (Actual $Y_{AA/CO}$ / Theoretical $Y_{AA/CO}$)	34.74%	45.41%
Specific CO uptake rate (mM CO/ hr/ g cells)	1.33	0.95
Specific H_2 uptake rate (mM H_2/ hr/ g cells)	0.34	0.26
Specific ethanol formation rate (mM ethanol/ hr/ g cells)	0.245	0.153

Table 5.3 Kinetic parameters of producer gas fermentations using P11 during agitation experiments.

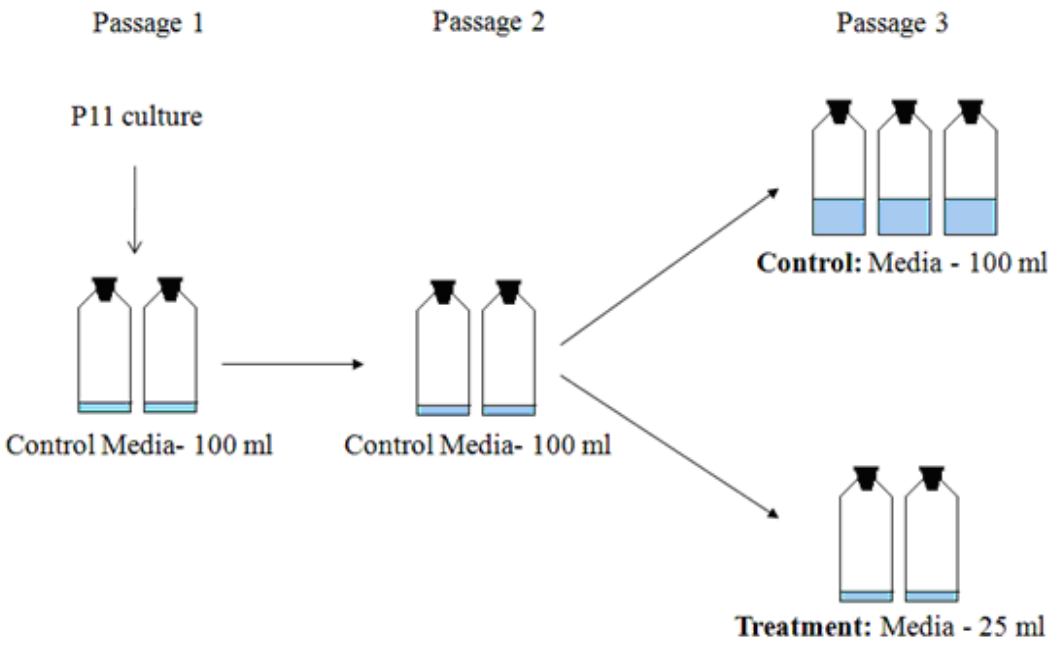

Figure 5.18 Experimental outline of headspace experiments

h but gas analysis and replacement of headspace gases was performed every 24 h. The experiment was conducted in triplicate with the control, but in duplicate (because of space limitation in the orbital shakers) with higher headspace. Experiment was stopped at 965 h because the product profiles with the increased headspace showed no signs of metabolic activity. From the observations by Frankman (2009), it was hypothesized that greater headspace would give higher cell growth and increased yields and productivities of ethanol.

Growth and pH: Cell mass concentrations and pH profiles are shown in Figs. 5.19 and 5.20, respectively. A 50 h lag phase in cell growth was observed in the treatment with regular headspace whereas rapid growth was observed with increased headspace. Maximum cell mass concentrations were 150% more with the increased headspace treatment with the regular headspace treatment. Increasing cell mass concentrations has been shown to increase reaction rates (Qureshi et al. 2005), which is crucial for producer gas fermentations. This could possibly be due to the presence of more gaseous substrates available with increased headspace.

Rapid decrease in pH was observed due to acetic acid production during the growth phase. With increased headspace, minimum pH was 4.6 while with normal headspace the minimum pH was 4.7. A slight increase in pH during solventogenesis was observed during solventogenic phase that could be due to acetic acid conversion into ethanol.

Product profiles: Ethanol and acetic acid production profiles are shown in Figs. 5.21 and 5.22, respectively. As previously observed acetic acid was a growth related product but unlike other fermentations ethanol was found to be a growth related product.

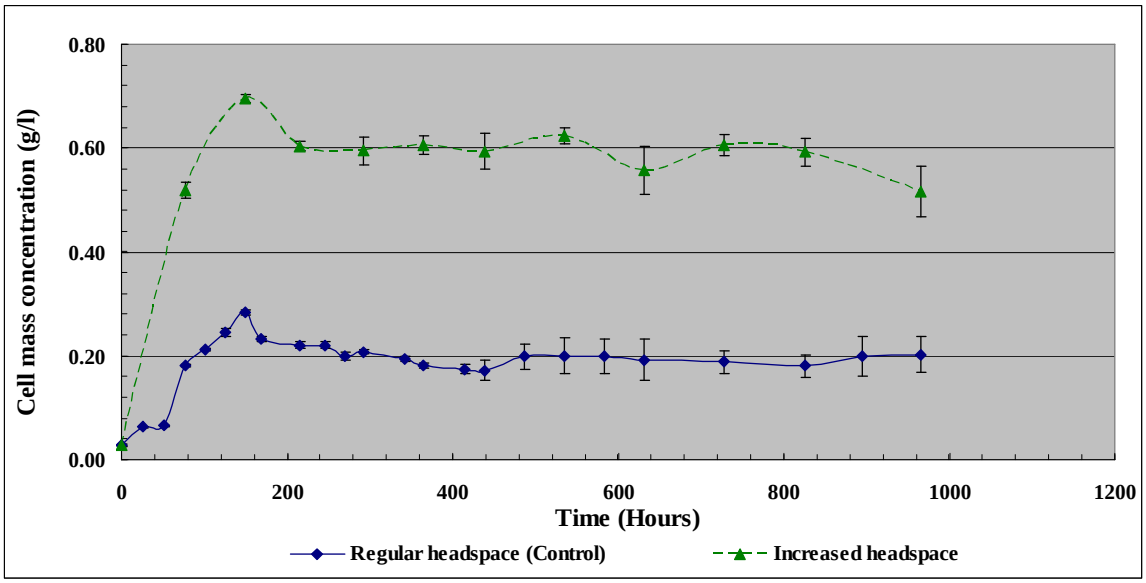

Figure 5.19 Cell growth in different treatments. Data points in control are the average of 3 replicates while the increased headspace is an average of 2 replicates; Error bars represent standard error.

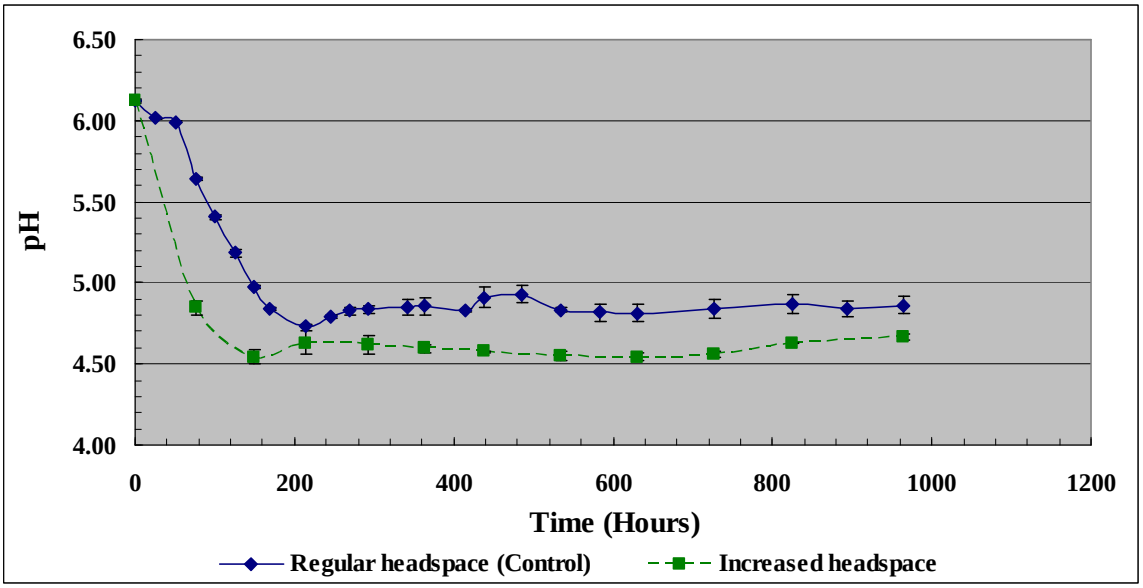

Figure 5.20 pH in different treatments. Data points in control are the average of 3 replicates while the increased headspace is an average of 2 replicates; Error bars represent standard error.

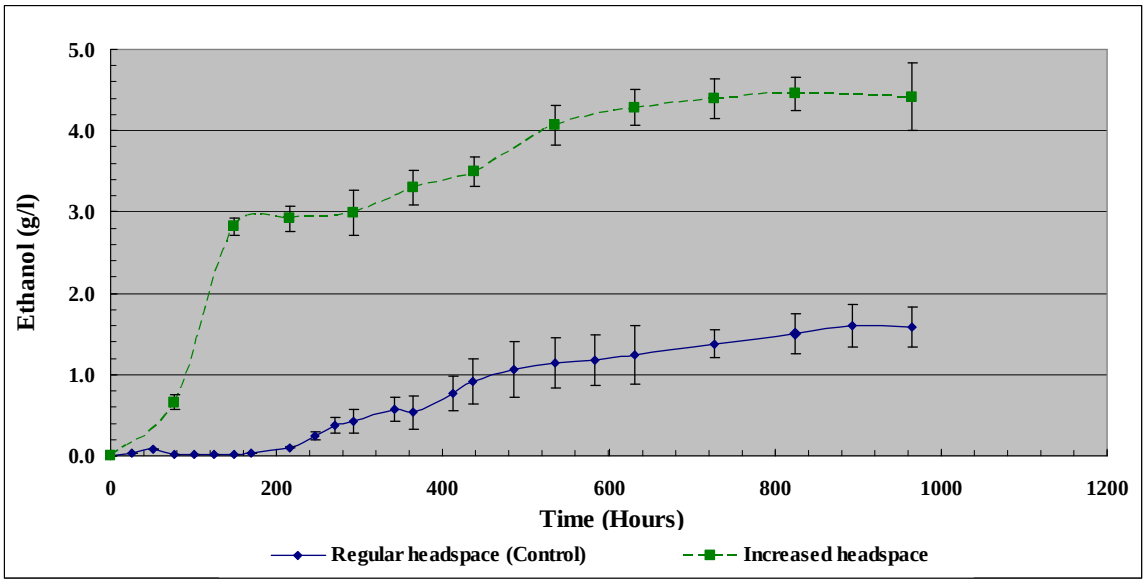

Figure 5.21 Ethanol production in different treatments. Data points in control are the average of 3 replicates while the increased headspace is an average of 2 replicates; Error bars represent standard error.

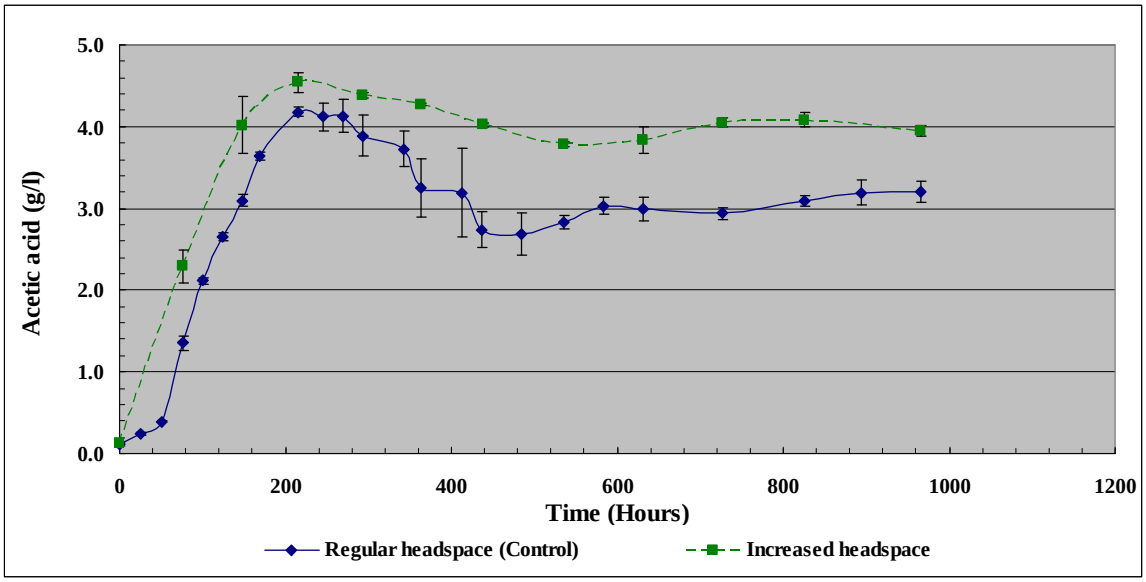

Figure 5.22 Acetic acid production in different treatments. Data points in control are the average of 3 replicates while the increased headspace is an average of 2 replicates; Error bars represent standard error.

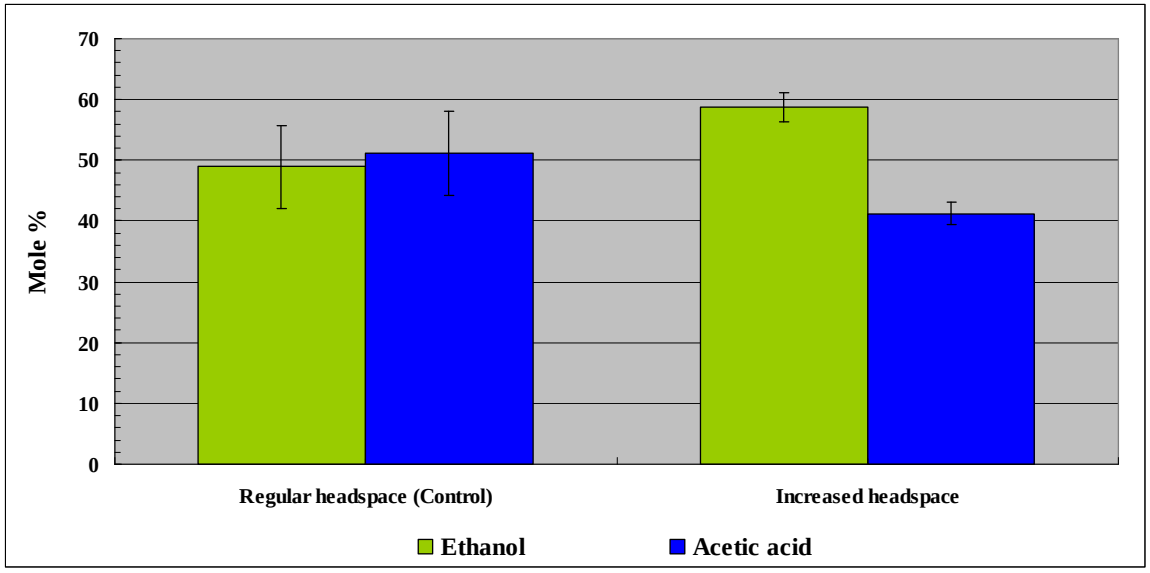

Figure 5.23 Molar percentages of products formed in different treatments. Data points in control are the average of 3 replicates while the increased headspace is an average of 2 replicates; Error bars represent standard error.

Formation of ethanol from CO and H_2 is thermodynamically favorable (Reaction 3.12 in chapter 3) and it is believed that proper environmental conditions would promote a growth related ethanol formation. With increased headspace a growth related ethanol was formed which could be due to the higher availability of gaseous substrates for ethanol formation. Rapid ethanol production was observed in the first 150 h followed by a pause in the production of ethanol for the next 140 h. Following this pause, ethanol production increased again as a non-growth related product. Ethanol production with the regular headspace started after 200 h and thus was a non-growth product. With increased headspace, 181% higher ethanol could be produced when compared to the control (4.5 g/l of ethanol was produced with increased headspace compared to 1.6 g/l with regular headspace). Solvent productivity was 865% higher with increased headspace, or in other words, it took 100 h to produce 1.6 g/l of ethanol with cells having more headspace while the same concentrations was produced in 965 h with the normal headspace.

Acetic acid concentrations increased rapidly with the growth of P11 cells in both treatments. Although, acetic acid concentrations were similar in both treatments it is important to note that only 6.3 g of acetic acid/ g cells was produced with the increased headspace compared to 14.7 g of acetic acid/ g cells with the regular headspace. Thus, lesser acetic acid per gram of cells was obtained using increased headspace which is important in our fermentations as far as product specificity is concerned. The acetic acid concentrations decreased slightly after they reached maximum concentrations at the end of growth phase, possibly due to the conversion of acetic acid into ethanol.

The ethanol to acetate molar ratio was 1.42 with increased headspace and 0.65 with the normal headspace. Thus, this research has shown that the molar percentages of

products (Fig. 5.23) can be altered using the amount of headspace in the bottle reactors that could be due to the increased availability of gaseous substrates for product formation. This is in accordance with Le Chatelier's principle which states that if equilibrium of a reaction is disturbed by changing the reaction conditions such as concentrations of the reactants, pressure and temperature, the position of the reaction moves to counteract the change (Myers 2003). In other words, if the concentrations of the reactants are increased, the rate of forward reactions will to also increase to form more products.

Gas consumption and kinetic parameters: The consumption of gaseous substrates such as CO, H_2 and CO_2 are shown in Figs. 5.24, 5.25 and 5.26, respectively. With a greater cell concentration with increased headspace, the CO and H_2 consumption and CO_2 production was higher than the cells that were grown with normal headspace. Presence of more gaseous substrates for the microbes by increasing the headspace resulted in increased gas uptake rates and higher ethanol formation rates (Table 5.4). However, after the growth phase, CO consumption decreased drastically, but marginal CO_2 consumption was observed with the treatment that had increased headspace. CO_2 consumption in the presence of CO could have occurred due to the necessity of a carbon source in the absence of CO consumption. Due to the decreased CO uptake during the stationary phase, the CO supplied remained unconsumed, which decreased the percentage of CO utilized. Further, since the volume of culture media was four times lesser than the control, the number of moles of ethanol was that was formed in the media was low, which decreased the yields and percent theoretical conversions drastically (Table 5.4).

The increased availability of gaseous substrates such as CO, CO_2 and H_2 due to the increased headspace, with higher cell yields would have contributed to presence of

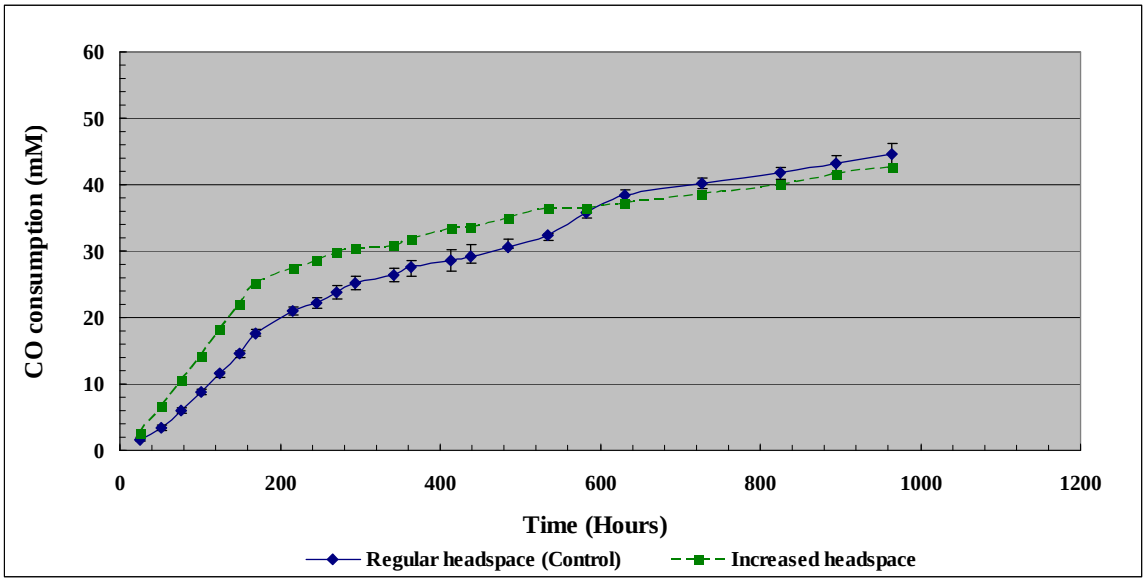

Figure 5.24 CO consumption in different treatments. Data points in control are the average of 2 replicates while the increased headspace did not have any replicates for gas analysis; Error bars represent standard error.

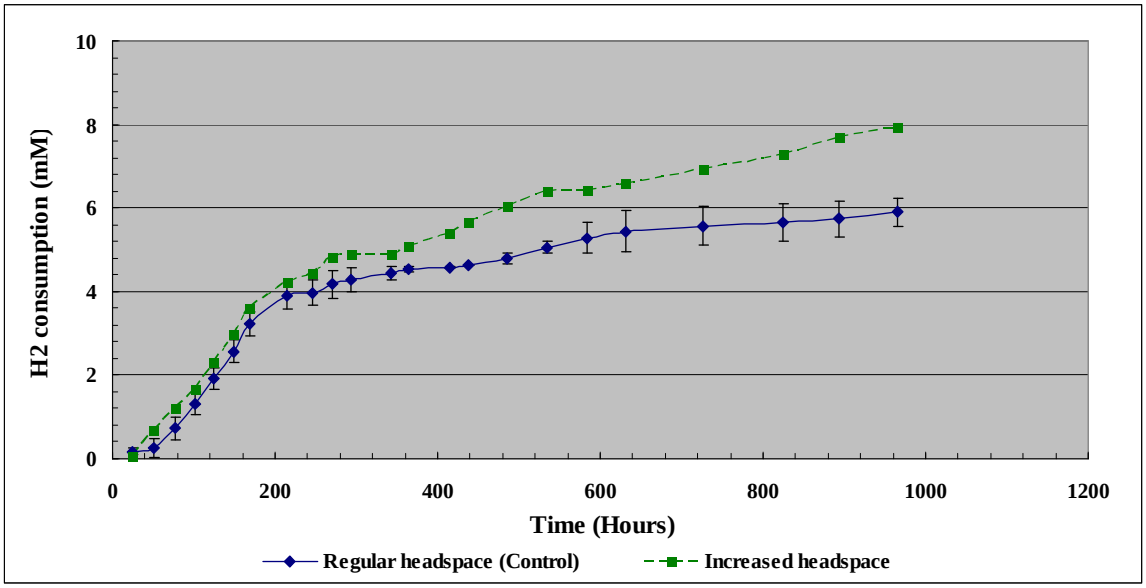

Figure 5.25 H_2 consumption in different treatments. Data points in control are the average of 2 replicates while the increased headspace did not have any replicates for gas analysis; Error bars represent standard error.

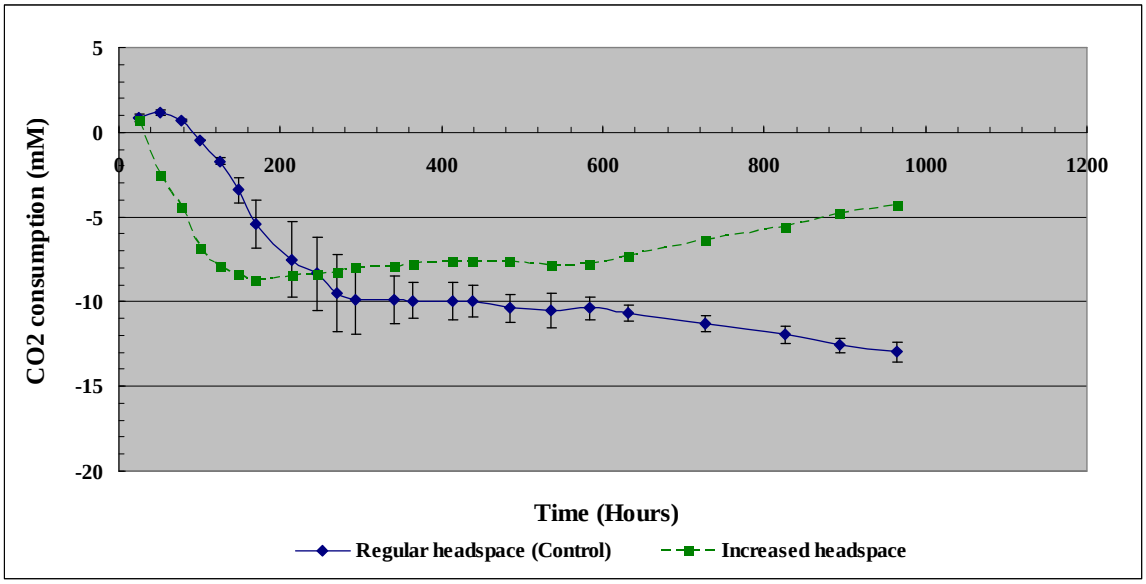

Figure 5.26 CO_2 consumption in different treatments. Data points in control are the average of 2 replicates while the increased headspace did not have any replicates for gas analysis; Error bars represent standard error.

Kinetic parameter	Regular headspace	Increased headspace
% CO utilized	45.96%	33.98%
% H_2 utilized	22.98%	25.24%
Yield (ETOH/CO) $Y_{ETOH/CO}$ (moles of ethanol/mole of CO)	0.062	0.031
Yield (AA/CO) $Y_{AA/CO}$ (moles of acetic acid/mole of CO)	0.111	0.029
% Theoretical conversion (Actual $Y_{ETOH/CO}$ / Theoretical $Y_{ETOH/CO}$)	17.49%	9.17%
% Theoretical conversion (Actual $Y_{AA/CO}$ / Theoretical $Y_{AA/CO}$)	22.11%	4.18%
Specific CO uptake rate (mM CO/ hr/ g cells)	4.78	7.22
Specific H_2 uptake rate (mM H_2/ hr/ g cells)	0.33	1.35
Specific ethanol formation rate (mM ethanol/ hr/ g cells)	0.207	0.600

Table 5.4 Kinetic parameters of producer gas fermentation using P11 during headspace experiments.

more reactants for the product formation in accordance to Le-Chatelier's principle. Frankman (2009) found that there was no change in mass transfer rates between serum bottles with 100 ml media and 50 ml media. Although, we employed 25 ml of media, further research is needed to confirm this assumption mass transfer benefit with the increase in headspace. The hypothesis of increased growth, ethanol productivities and higher ethanol concentrations resulting from increased headspace was successfully demonstrated.

5.4 Conclusion

This research has found the effect of physical parameters such as headspace gas volume, agitation rates and heat shock treatment on ethanol concentrations in producer gas fermentations using P11 cells in lab scale bottle reactors. Both the temperature shocks at 75°C and 92ºC for 3 min increased gas uptake rates and initiated an early induction of solventogenesis, but 92ºC heat treatment resulted in an increase in ethanol (55%) and acetic acid (13%) when compared to the control that was not heat shocked. The increase in agitation rates from 150 rpm to 250 rpm decreased ethanol (50%) and acetic acid (52%), possibly due to the shear sensitivity of cells. The increase in headspace volume resulted in a 181% increase in ethanol concentration and 23% increase in acetic acid concentration. Besides this, ethanol was produced as a growth related product with increased headspace volume that eventually increased the productivity of the ethanol production by 865%. Thus, heat shocking of P11 cells and increasing the headspace volume has shown promising results with increases in ethanol to acetate ratios and ethanol productivities.

Acknowledgements

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CHAPTER 6

FUTURE WORK

This research has provided an understanding on the effects of producer gas and its components such as methane and oxygen on *Clostridium* strain P11 fermentations. During the study, the robustness of the microbe was shown with the components of producer gas generated by pilot scale gasifiers. However, further studies have to be performed for the successful integration of gasification and fermentation process. The following studies are considered essential.

- The cause of enhanced solvent formation during the fermentation of producer gas generated from the gasifiers must be determined. There could be multiple components such as nitric oxide (Ahmed and Lewis 2007) in the producer gas that contribute to this increase in solvent yields. The effect of individual components must be studied closely. Enzymatic assays for essential enzymes such as FDH, CODH, ADH and hydrogenase will give answers to the metabolism of the microbe. The study should then be integrated to determine whether the producer gas constituents have an additive effect on fermentation. This would help in developing the gas cleaning technologies based on the tolerance of producer gas components to keep the cost of the process to the minimum level.

- Isopropanol which was a major product formed during switchgrass producer gas experiments could have been formed by the reduction of acetone. This study has determined that acetone could act as a major contaminant of producer gas fermentations (Appendix B) and thus methods have to be developed for complete removal of acetone from producer gas. Since, the solubility of acetone is high; it could be removed by bubbling through water. Although, it is believed that isopropanol is produced from the reduction of acetone, it could also be produced by the reduction of propionic acid. Hence, experiments will have to be conducted for the evaluation of metabolic pathway of isopropanol production from P11.
- Heat shocking of P11 cells at 92ºC for 3 min showed increased solvent concentrations. Although spores were detected after heat shocking, microbial staining of cells were not performed during the different stages of the P11 fermentation. P11 cells could be sporulating and/or also forming heat shock proteins and has to be studied closely to see if there is any relation between sporulation and heat shock protein expression with solvent formation.
- In our studies, the availability of gaseous substrates increased the rate of ethanol formation. Thus, it is believed that gas mixtures with composition close to stoichiometric ratios will increase the amount of cells, rate of product formation than the synthetic gas mix such as 20% CO, 15% CO_2 and 5% H_2. Effect of different gas compositions will help in understanding the metabolism of the microbe.

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APPENDIX A

Calculation of kinetic parameters

% Gas utilization

In producer gas fermentations, CO acts as a source of carbon and electrons while H_2 serves as an electron source. Improving the consumption of both CO and H_2 would improve the fermentation yields and hence they are one of the most important kinetic parameters. In batch reactors, the gaseous substrates were fed periodically at an interval of x hours (h). The molar percentage of gas in inlet and after x h was calculated using the GC-TCD. Ideal gas law was used to convert the gas percentages into number of moles. The difference between the moles of CO in the inlet and moles of CO after x h would equal the moles of CO consumed by the microbe in that particular time (x h).

$$CO_{in} = \frac{P_i V y_i}{RT}$$

$$CO_{out} = \frac{P_f V y_f}{RT}$$

Moles of CO consumed in x h = CO_{in} - CO_{out}

Total moles of CO consumed = Sum of moles of CO consumed at every x hours.

Total moles of CO provided = Sum of moles of CO provided after every x hours.

% CO utilized= (Total moles of CO consumed/ Total moles of CO provided)*100

Where,

P_i = Inlet pressure of the batch reactor.

P_f = Pressure of the batch reactor after x hours.

V = Total volume of the gas in the reactor.

y_i = Initial mole fraction of CO in batch reactor.

y_f = Mole fraction of CO in batch reactor after x hours that is calculated by the GC-TCD.

R = Molar gas constant.

T = Temperature in Kelvin.

Similarly,

% H_2 utilized= (Total moles of H_2 consumed/ Total moles of H_2 provided)*100

Yield of ethanol ($Y_{ETOH/CO}$) and Yield of acetic acid (Y_{AA}/CO)

Concentrations of ethanol and acetic acid produced from the fermentations were calculated by the GC-FID. From the concentrations, moles of ethanol and acetic acid formed during fermentation are calculated but the formula (moles= g of product/ molecular weight of the product). Total moles of CO consumed were calculated as described in the previous section. CO is the limiting substrate for our reactions because in the absence of CO, no products will be formed.

Thus,

$Y_{ETOH/CO}$ = Total moles of ethanol produced/ Total moles of CO consumed

Similarly,

$Y_{AA/CO}$ = Total moles of acetic acid produced/ Total moles of CO consumed

% Theoretical conversion of ethanol and acetic acid

The theoretical maximum yield of ethanol and acetic acid is calculated from the reactions:

$3CO + 3\ H_2 \rightarrow\ C_2H_5OH + CO_2$

$2\ CO + 2\ H_2 \rightarrow CH_3COOH$

It could be observed that 1 mole of ethanol can be formed from 3 moles of CO, thus making the theoretical maximum yield of ethanol as 0.333. Similarly, from the second reaction, 2 moles of CO is required for the formation of 1 mole of acetic acid. Thus, the theoretical maximum yield of acetic acid is 0.5.The % theoretical conversion of the product is the ratio of actual yields of product observed in our fermentations (which are calculated from the last section) to the theoretical maximum yields of the product. Mathematically,

% Theoretical conversion of ethanol = Actual $Y_{ETOH/CO}$/ Theoretical $Y_{ETOH/CO}$

where, Actual $Y_{ETOH/CO}$ was calculated from last section and theoretical $Y_{ETOH/CO}$ is 0.333.

Similarly,

% Theoretical conversion of acetic acid = Actual $Y_{AA/CO}$/ Theoretical $Y_{AA/CO}$

where, Actual $Y_{AA/CO}$ was calculated as described before and theoretical $Y_{AA/CO}$ is 0.5.

Specific CO and H_2 uptake rates (q_{CO} and q_{H2})

Gas uptake rate is defined as the amount of gas consumed per unit time per unit mass of cells. Thus,

$$q_{CO} = (CO_{in} - CO_{out}) / (\text{g cells in the bioreactor} * t)$$

where,

CO_{in} - CO_{out} can be calculated as described before.

g cells in the bioreactor= Product of cell mass concentration (X) and volume (Vega et al.) of culture media in the batch reactor.

t = Time taken to consume total moles of CO during the fermentation.

Similarly,

$$q_{H2} = (H_{2in} - H_{2out}) / (\text{g cells in the bioreactor} * t)$$

where,

$H_{2in} - H_{2out}$ can be calculated as described before.

Specific ethanol formation rates (q_{ETHOH})

Specific ethanol formation rate is defined as the ratio of molar ethanol production rate per unit time per unit mass of cells. Thus,

$$q_{ETOH} = (\text{moles of ethanol produced/ time}) / (\text{g cells in the bioreactor})$$

where,

moles of ethanol produced is calculated from GC-FID

Time = (Total fermentation time – lag time for ethanol formation). This lag time is subtracted because ethanol is a non growth related product and no ethanol is usually observed in the first 200 h of fermentation (approximately).

g cells in the bioreactor = Product of cell mass concentration (X) and volume (Vega et al.) of culture media in the batch reactor.

APPENDIX B

Corn gluten feed experiment in the presence of 1.63% oxygen

Introduction

Oxygen is one of the most toxic gases to acetogens because many of the enzymes present in the acetyl-CoA pathway are extremely sensitive to oxygen (Drake et al. 2006). Oxygen has been reported during biomass gasification in the past (Datar 2003). Datar (2003) studied the effect of oxygen on *Clostridium carboxidivorans* and found that oxygen concentrations up to 1900 ppm (or 0.19%) did not affect CO and H_2 utilization, growth and product formation in *C. carboxidivorans*. Several acetogens like *Acetobacterium woodii , Clostridium magnum, Sporomus silvatica, Moorella thermoautotrophicum* and *Clostridium glycolicum* RD-1 have shown the tendency to tolerate oxygen concentrations in the range of 0.5%-6% as they contain enzymes involved in the removal of oxygen (or its toxic products) such as NADH-oxidase, peroxidase, superoxide dismutase, rubredoxin oxidoreductase and rubrerythrin (Drake et al. 2006; Karnholz et al. 2002; Kusel et al. 2001).

The objective of this research was to study the effect of presence of 1.63% oxygen in the fermentation of a corn gluten feed (CGF) generated producer gas by *Clostridium* strain P11.

Materials and Methods

All materials and methods are similar to that described in Chapter 4. The media composition used for the cultivation of P11 was the same as that was used for the methane experiments.

Results and discussion

Batch studies- Effect of CGF producer gas in the presence of 1.63% oxygen

Producer gas was generated from a downward draft gasifier with CGF as substrate and a gas composition as shown in Table B.1 was obtained. CGF is a byproduct of the corn wet milling industry. The CGF producer gas also had oxygen at 1.63% due to the compressor malfunction which added atmospheric oxygen into the gas storage tanks. The presence of oxygen could be toxic to many enzymes of the acetyl-CoA pathway (Drake et al. 2006). Preliminary studies using switchgrass producer gas with 1.27% oxygen showed acetone as a major product of fermentation at concentrations as high as 1.62 g/l in 10 days with cell mass concentrations as high as 0.5 g/l (data not shown). This was a promising result because there were no previous reports on acetone production from P11. However, neither acetic acid nor ethanol was observed in the preliminary study. Thus, research questions like “whether P11 produced acetone and was this product distribution an effect of presence of oxygen in producer gas” or “whether there is some other microbe (contaminated culture) which produced acetone” had to be answered. Microscopic evaluation of the preliminary culture revealed the presence of cocci shaped cells (while P11 are rod shaped) in the bottles that produced acetone, which suggested that the fermentation might have been contaminated by some other microorganism. The contaminated culture is hereafter referred as “acetone culture”.

Gas Type	Producer gas composition (%)
Carbon monoxide (CO)	10.08
Carbon dioxide (CO_2)	8.94
Hydrogen (H_2)	8.50
Methane (CH_4)	1.85
Acetylene (C_2H_2)	0.10
Ethylene (C_2H_4)	0.44
Ethane (C_2H_6)	0.10
Nitric oxide (NO)	ND
Oxygen (O_2)	1.63
Nitrogen (N_2) (Balance gas)	68.36

Table B.1 Producer gas compositions from on a on a downdraft reactor with CGF as feedstock; ND- not determined; Gas composition reported is the average of three injections on Gas chromatography (GC) with a Thermal conductivity detector (TCD).

Also, it was important to determine whether the observed acetone was biologically produced or was a contaminant of biomass producer gas. This question was raised because a 20% acetone solution was used in the scrubbers to reduce the amount of tars in the gas. It could be possible that a part of this acetone accumulates in the producer gas during scrubbing process.

The outline of the experiment is shown in Fig. B.1. Both P11 and the acetone culture were tested for growth and product formation on synthetic gas mix and CGF producer gas. The hypothesis was that P11 did not make acetone and it formed either by the contaminated culture or abiotically. To test whether acetone was produced abiotically two sets of negative control were added to the experiments. The negative controls were treated the same way as the treatment bottles except they did not have any cells inoculated. One set of duplicate bottles had synthetic gas mix and the other set had CGF producer gas. In either of the negative controls, no cell growth was expected. If acetone was present in the producer gas generated from the gasifiers, it will get accumulated in the liquid media and thereby our hypothesis can be proved. If contaminated culture produced acetone in both synthetic gas mix and CGF producer gas then we could prove that the contaminated cocci cells were responsible for the production of acetone.

Growth and pH: The growth and pH profiles are shown in Fig. B.2 and B.3. In all the graphs, the negative control with CGF producer gas was run the shortest time (168 h) because the batch of CGF producer gas was all consumed during the experiments and no feedstock (CGF) was available for gasification. All the experiments other than the control (denoted as P11: synthetic gas) were stopped at 336 h because there was not much change in the concentration of products like ethanol and acetic acid. The control

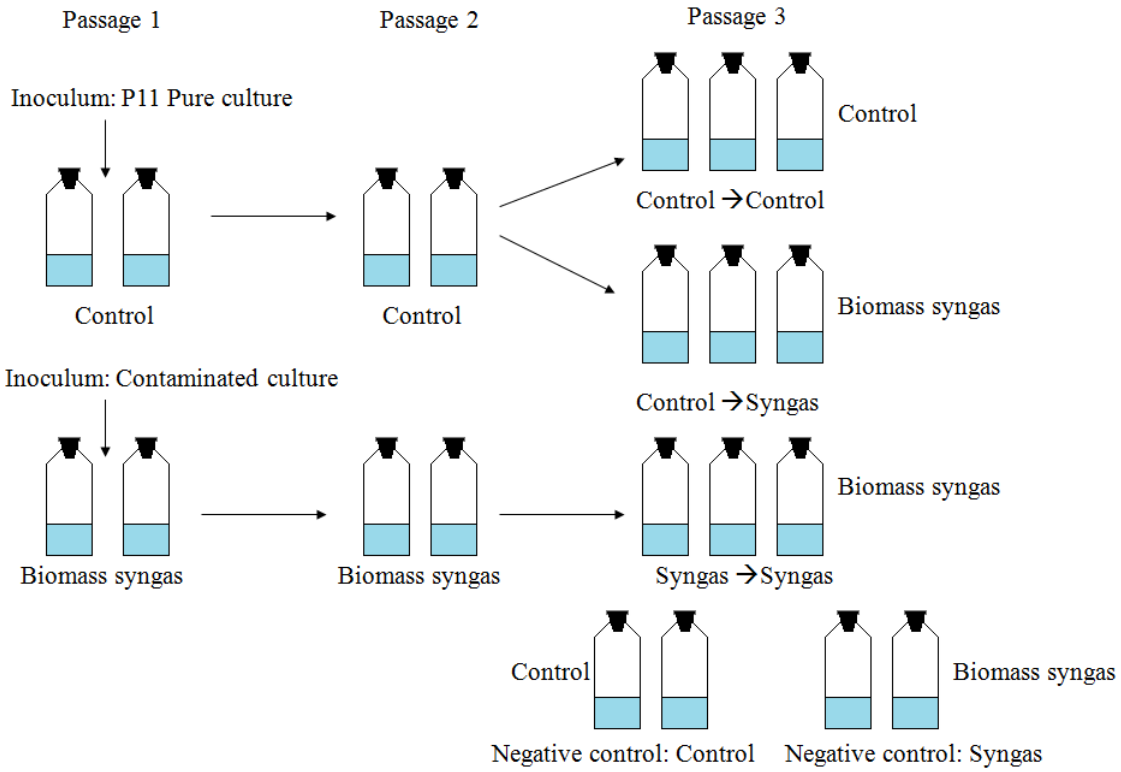

Figure B.1 Experimental outline for CGF producer gas experiment; Control-Synthetic gas mix of composition 20% CO, 15% CO_2, 5% H_2 and 60% N_2 as substrate; Biomass syngas (or syngas) - CGF producer gas of gas composition shown in Table B.1 as substrate; Control → Control- Cultures grown on control gas mix throughout the experiment (Passage 1, 2 and 3). This serves as a control for the whole experiment; Control → Syngas- Culture that was grown in synthetic gas mix (in Passage 1 and 2) are transferred and grown on producer gas (in passage 3); Syngas→ Syngas- Cultures that were grown in producer gas throughout the experiment (Passage 1, 2 and 3); Negative control: Control- Uninoculated bottle reactor with control gas mix; Negative control: Syngas- Uninoculated bottle reactor with CGF producer gas.

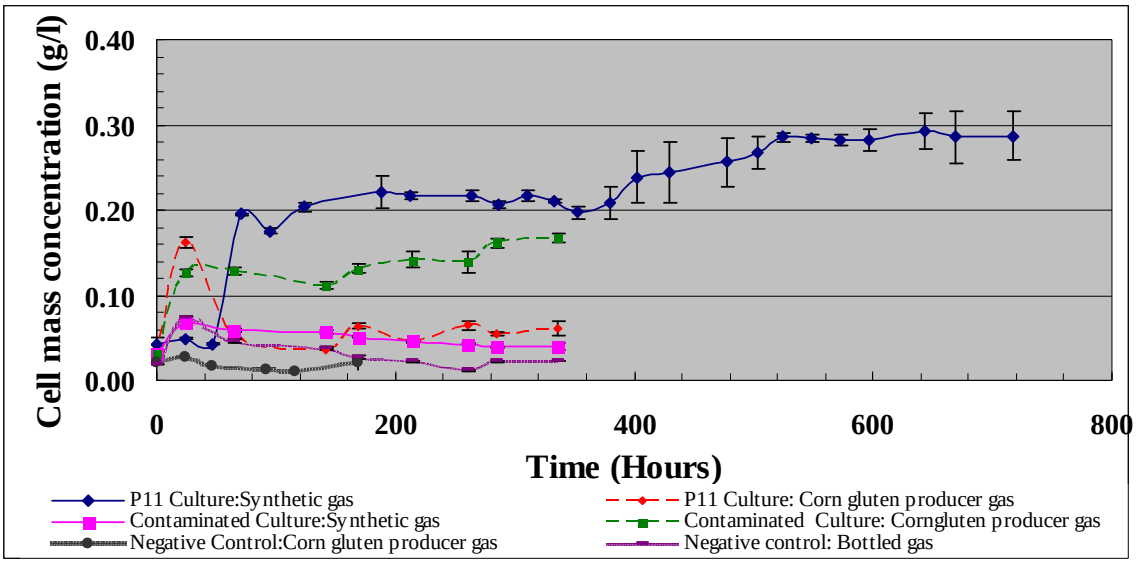

Figure B.2 Cell growth in different treatments; Each data point is the average of number of replicates; Number of replicates in each treatment is 3 except the negative controls where the number of replicates is 2; Error bars represent standard error.

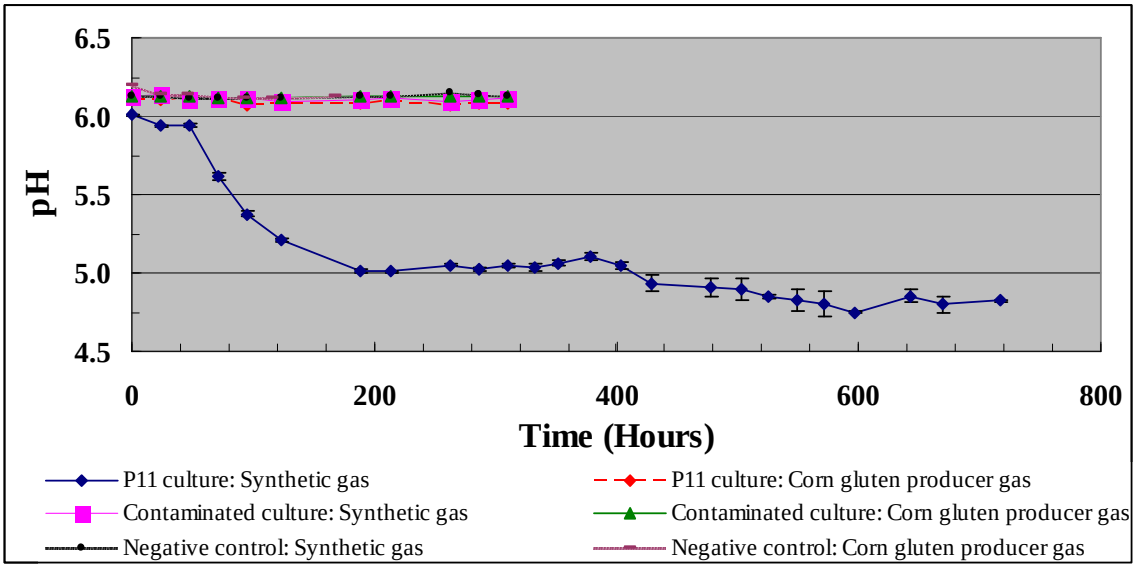

Figure B.3 pH profile in different treatments; Each data point is the average of number of replicates; Number of replicates in each treatment is 3 except the negative controls where the number of replicates is 2; Error bars represent standard error.

(denoted as P11: synthetic gas) was stopped when the product formations leveled off at 720 h.

The only treatment where there was cell growth is the control (P11 culture on synthetic gas) and the contaminated culture (cocci cells) on CGF producer gas. The control had a lag time of 47 h after which exponential growth was observed. As reported earlier, pH was observed to decrease during the growth phase. In control, the cells reached the stationary phase at 123 h, and after 350 h increase in cell mass concentrations were observed again. This second growth phase was linked with an increase in acetic acid concentration, decrease in pH and higher uptake of substrates like CO and H_2 gases. Multiphase growth similar to our observations were also observed in the microbe *Clostridium acetobutylicum* (Tracy et al. 2008).

P11 cells grown on CGF producer gas in the presence of oxygen showed a sharp increase in cell concentrations (reached 0.16 g/l) in the first 24 h, but after that there was a steep decrease in cell mass concentration to 0.06 g/l cells. This increase in cell mass could be due to the removal of oxygen by cysteine sulfide solution, which is added to fermentations to scavenge traces of oxygen. The resazurin in media remained colorless for the first 24 h of fermentation, showing the ability of the reducing agent (cysteine sulfide) to reduce oxygen to tolerable limits for the growth of the microbe. After 24 h, the color of the media remained pink for the rest of the fermentation, showing the presence of oxygen in the media. The decline in cell mass concentration indicates that 1.63% of oxygen was toxic to the cells. This shows that P11 possibly does not have enzymes like NADH-oxidase, peroxidsase, superoxide dismutase, rubredoxin oxidoreductase and

rubrerythrin to detoxify the harmful radicals produced in the presence of oxygen which are reported in some studies (Drake et al. 2006; Karnholz et al. 2002; Kusel et al. 2001).

No change in the pH or acid production was observed in any levels of treatments except the control (Fig. B.3).

Product profiles: Ethanol, acetic acid and acetone concentrations over time are shown in Figs. B.4, B.5 and B.6, respectively. Ethanol production was only observed in the control (P11 culture grown on synthetic gas). Ethanol was a non growth associated product with an exponential increase in production only after 330 h. The standard error bars are larger towards the end of fermentation due to variation between replicated bottles.

Acetic acid was also found to be produced only in control (P11 culture grown on synthetic gas). Acetic acid production was growth related as ATP is generated by substrate level phosphorylation during its production. A slight increase in acetic acid concentration (~1 g/l) was observed after 400 h, which was directly related to cell growth, CO and H_2 consumption. The increase in acetic acid production could be due to increasing demand of ATP inside the cell for its survival and other metabolic activities (Meyer and Papoutsakis 1988). No acetic acid and ethanol was formed in the treatments when P11 culture was grown on CGF producer gas, which confirms that 1.63% oxygen was toxic to product formation.

Acetone accumulated in the treatments (both P11 culture and contaminated culture) using CGF producer gas. However, it was also observed in negative controls. The presence of acetone in negative controls is likely due to the presence of acetone in producer gas from the acetone scrubber used to clean the gas. Since acetone is highly

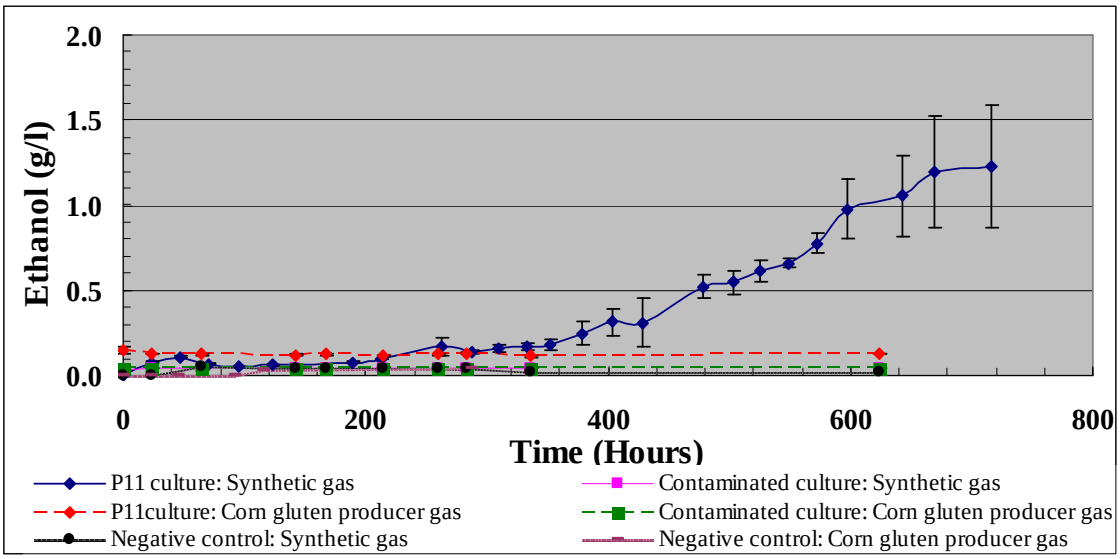

Figure B.4 Ethanol production in different treatments; Each data point is the average of 3 replicates; Error bars represent standard error; Number of replicates in each treatment is 3 except the negative controls where the number of replicates is 2.

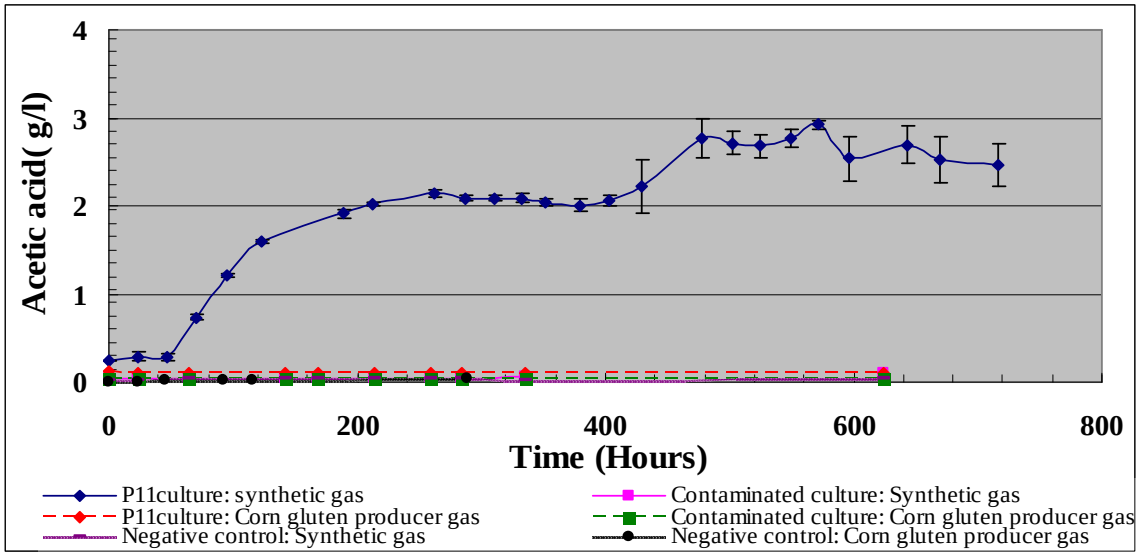

Figure B.5 Acetic acid production in different treatments; Each data point is the average of 3 replicates except the negative controls where the number of replicates is 2; Error bars represent standard error.

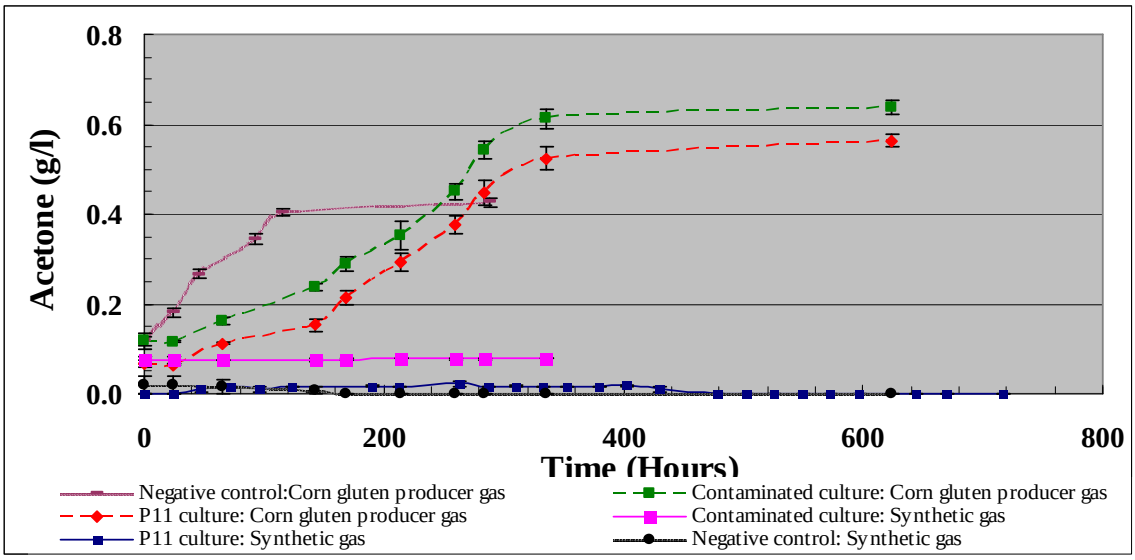

Figure B.6 Acetone production in different treatments; Each data point is the average of 3 replicates except the negative controls where the number of replicates is 2; Error bars represent standard error.

soluble in water, it dissolved from the gas into liquid media. To confirm this, gas samples were analyzed in a GC-MS (Gas chromatography- mass spectrometer) column which showed the presence of acetone in CGF producer gas. However, when gas samples were taken from a reactor which had liquid media, no acetone was observed in the headspace (data not shown). Even when de-ionized (DI) water was used instead of regular liquid media, no acetone was observed in the headspace (data not shown). However, acetone was observed when the liquid samples were analyzed using a GC-FID. This supports our hypothesis that acetone in the liquid media was the result of acetone in the producer gas and not biological production. Furthermore, when no addition of producer gas was made at 116 h for negative control and 336 h for contaminated culture and pure culture grown on CGF producer gas, they showed no increase in acetone production, which further confirms our findings. Acetone is not produced during gasification of biomass feedstocks but was carried over from the scrubbing system which is employed for the removal of tars from producer gas.

The contamination of acetone into liquid media could interfere with producer gas fermentations in many ways as it could: a) give misleading information about product formation and b) give rise to other metabolic products from acetone such as isopropanol as acetone is a substrate for isopropanol production (as shown in Fig. 4.1). CGF producer gas fermentation did not produce any isopropanol as the major metabolic activities were shutdown due to the presence of oxygen. However, switchgrass producer gas did produce isopropanol at concentrations as high as 3.9 g/l. Although no acetone was observed during the analysis of the switchgrass producer gas fermentations, the GC method used was unable to detect acetone. Therefore, the possibility of acetone reduction into

isopropanol cannot be ruled out since P11 was able to reduce acetone into isopropanol in the presence of glucose as carbon source (data not shown). Further research needs to be done to confirm whether acetone can be biologically produced by P11 and whether the acetone conversion occurs in the presence of CO, CO_2 and H_2 as gaseous substrates.

Gas consumption profiles: The consumption of CO, H_2 and CO_2 are shown in Figs. B.7, B.8 and B.9, respectively. In control bottles (P11 culture grown on synthetic gas mix) CO consumption started sharply after 47 h of lag phase and slowed down during the stationary phase (from ~100 h to 350 h). After 350 h, there was an increase in the rate of CO consumption due to a secondary growth phase which produced acetic acid as well (Fig. B.7). The H_2 consumption data (Fig. B.8) had the same trend as the CO data (Fig. B.7) implying that CO and H_2 serve as two substrates for acetic acid and ethanol formation possibly from the theoretical reactions described as follows (Barik et al. 1988; Phillips et al. 1994; Ragsdale 1991; Rajagopalan et al. 2002).

$$2CO + 4H_2 \rightarrow C_2H_5OH + H_2O \quad \text{(B.1)}$$

$$CO + H_2O \rightarrow CO_2 + H_2 \quad \text{(B.2)}$$

$$3CO + 3\,H_2 \rightarrow C_2H_5OH + CO_2 \quad \text{(B.3)}$$

$$2CO + 2H_2 \rightarrow CH_3COOH \quad \text{(B.4)}$$

$$CH_3COOH + 2\,H_2 \rightarrow C_2H_5OH + H_2O \quad \text{(B.5)}$$

$$5CO + 7H_2 \rightarrow 2C_2H_5OH + CO_2 + H_2O \quad \text{(Overall reaction)} \quad \text{(B.6)}$$

The gas analysis data shows that P11 has a preference for CO as a carbon source when a mixture of CO and CO_2 are provided as carbon source. Other acetogens like *P. productus* (Vega et al. 1988a) and *C. carboxidivorans* (Shenkman 2003) have also shown preference to consume CO when a mixture of CO, CO_2 and H_2 are provided. On the CO_2

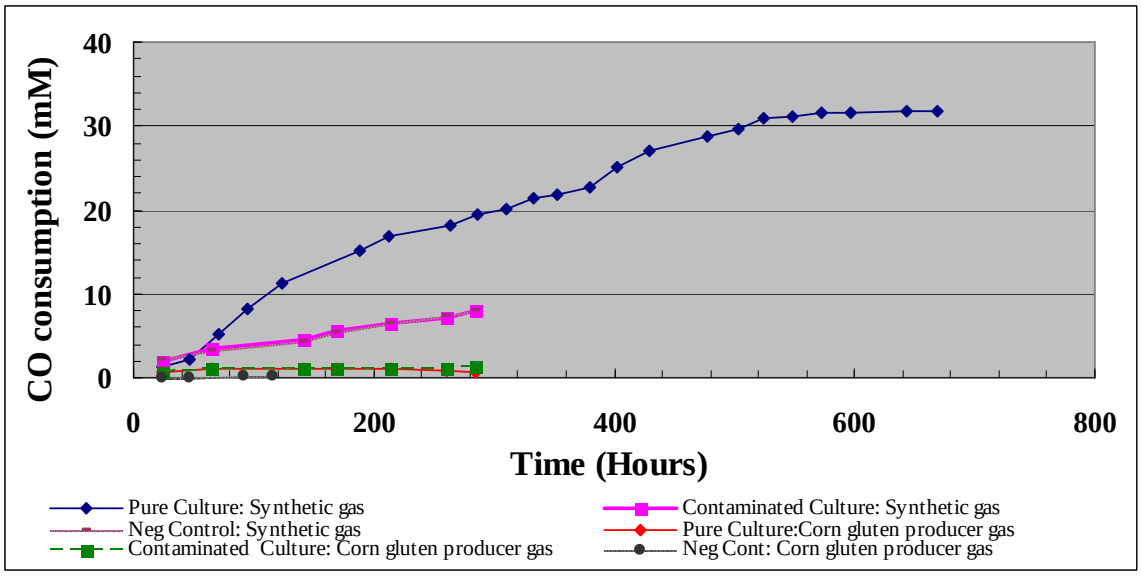

Figure B.7 CO consumption in different treatments.

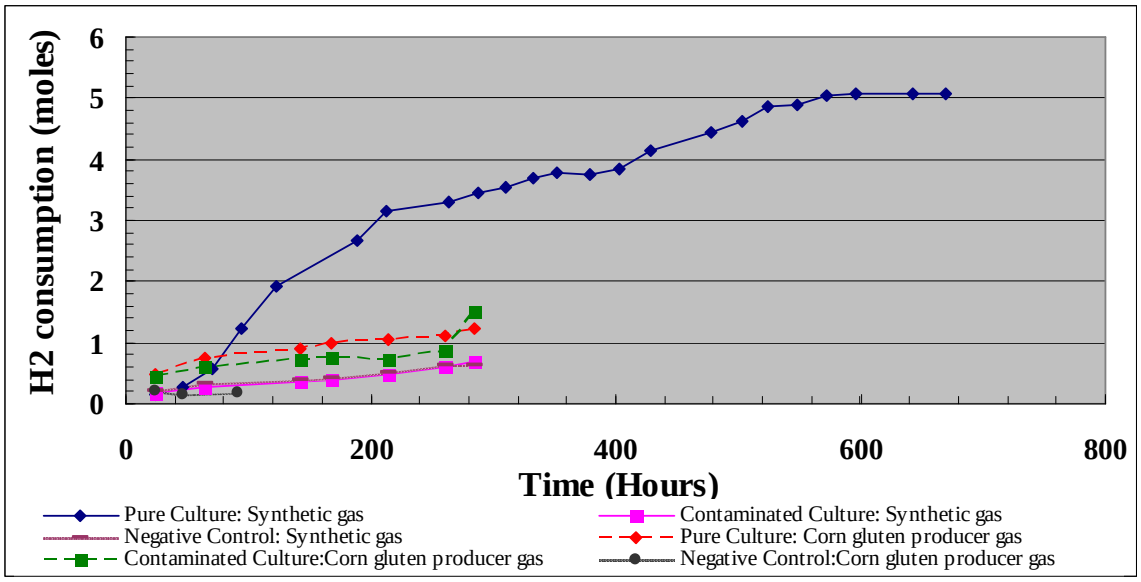

Figure B.8 H_2 consumption in different treatments

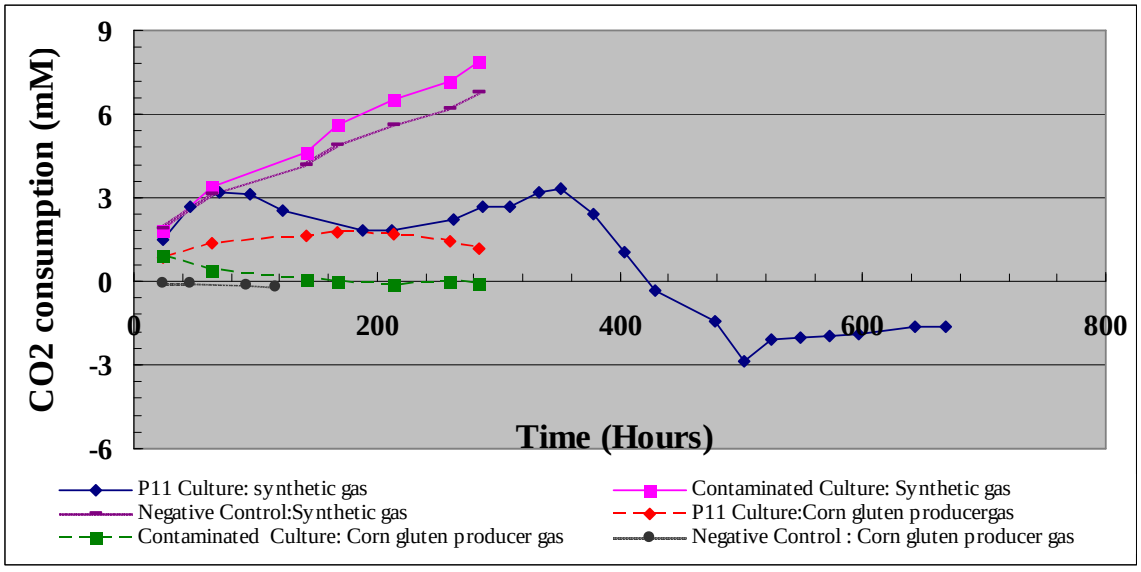

Figure B.9 CO_2 consumption in different treatments.

consumption profile (Fig. B.9), no CO_2 consumption occurred during the lag phase followed by a production phase from 79 h to 189 h, a consumption phase from 189 h to 350 h, a secondary production phase from 350 h to 503 h and then no consumption or production was observed after 503 h.

In Fig. B.7, it could easily be seen that contaminated culture and negative controls on synthetic gas overlap each other and the small increase in CO could be due to its solubility in the media. A similar trend was observed in contaminated culture and negative controls on CGF producer gas. The variation between the treatments grown on synthetic gas and CGF producer gas could be due to the difference in the concentration of CO in the two gaseous substrates (as synthetic gas had 20% CO whereas CGF producer gas had only 10% CO). Similar variation was also seen in hydrogen consumption curves because of the difference in concentration of H_2 in the two gaseous substrates (as synthetic gas had 5% H_2 whereas CGF producer gas had 8.9% H_2). CO_2 production and H_2 consumption were seen with the treatment in which contaminated culture was grown on CGF producer gas.

The contaminated cocci culture was later discovered to be a *Staphylococcus* species (determined by 16 S rRNA sequencing) and was observed to be a facultative anaerobe and was unable to grow on $CO/CO_2/H_2$ as the sole carbon and energy source (Delorme and Wilkins, Presentation at SIM Annual meeting and exhibition, 2009).

Conclusion

In this study, we demonstrated that 1.63% oxygen was toxic to P11 as it did not show any growth. Further investigations would need to be carried out to find out the tolerance limit of oxygen by P11. Nevertheless, this research identified the presence of

acetone as a producer gas contaminant from scrubbing system and hence better way to remove or reduce the concentrations of acetone from producer gas will have to be identified.

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VITA

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Candidate for the Degree of

Master of Science

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Major Field: Biosystems Engineering

Scope and Method of Study:

The effect of producer gas generated by gasification of Kanlow switchgrass was determined to investigate the robustness of *Clostridium* strain P11 for the integration of gasification-fermentation process in a lab scale. Further experiments were performed for determining the effect of 5% methane, as methane is a major component of producer gas generated through gasification. Effect of 1.63% oxygen that was present in corn gluten feed (CGF) producer gas was also determined. Furthermore, the effect of physical parameters such as heat shocking, agitation and headspace gas (amount of available gas) were determined for enhancing the solvent concentrations and productivities.

Findings and Conclusions:

The use of switchgrass producer gas generated from fluidized bed gasifier showed an increase in ethanol concentrations by 125%, decrease in acetic acid concentrations by 40% and production of isopropanol at concentrations as high as 3.9 g/l. Producer gas components such as tars, nitric oxide and hydrocarbons could possibly be responsible for the metabolic shifts. 5% methane did not affect growth, product formation and gas uptake in P11 fermentation indicating that methane was an inert component of producer gas. However, the presence of 1.63% oxygen was highly toxic to P11 cells as it inhibited growth, product formation and gas uptake rates. Also, acetone that was used in gas-clean up was found to be contaminant of producer gas in the CGF experiment as it was getting accumulated in the culture media.

Increasing agitation rates inhibited product formation possibly due to the shear sensitivity of P11. Heat shocking at 92°C improved the percentage of gas (CO and H_2) utilization, yields of ethanol and acetic acid, ethanol productivity when compared to cells that were heat shocked at 75°C and the cells that were not heat treated. By increasing the headspace gas, cell mass and ethanol concentrations increased by 160% and 181% respectively and the ethanol productivity increased by 865% when compared to the cells that were grown with the normal headspace.

ADVISER'S APPROVAL: Dr. Mark R. Wilkins